# MAGNESIUM IN THE NUTRITION OF THE RABBIT[1]

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FIVE FIGURES

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The symptoms developed during magnesium deprivation in the rat have been well described (Kruse, Orent and McCollum, '32; Tufts and Greenberg, '38). Because of the anatomical and physiological character of herbivorous animals, their dietary requirements are somewhat different from those of the rat (Olcese, Pearson and Schweigert, '48).

The development of a purified diet supporting nearly normal growth in the rabbit, which has a herbivorous dietary habit (Wooley and Sebrell, '45), and the small size of this animal have made possible the further study under laboratory conditions of the dietary requirements of herbivora. The studies reported here were conducted in order to ascertain the effect of magnesium deficiency on rabbits and to obtain information on the quantitative requirement of these animals for magnesium.

## EXPERIMENTAL

Weanling New Zealand white rabbits of both sexes, 5 to 6 weeks of age and weighing from 0.6 to 1.3 kg, were used in these experiments. Food and distilled water were given ad

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libitum except when the rabbits became diarrheal, at which times the water supply was limited to about half that normally consumed. The rabbits were weighed weekly during the experimental period and were confined to wire-bottom cages in groups of three or 4.

Blood samples were obtained from the rabbits by cardiac puncture without anesthesia. The plasma and whole blood magnesium were determined by the method described by Kunkel, Pearson and Schweigert ('47). Serum calcium was determined by the method of Kramer and Tisdall ('21) as modified by Clark and Collip ('25). Hematocrit readings were made with the use of Wintrobe hematocrit tubes centrifuging at about 3500 r.p.m. for one hour.

As a result of the simultaneous investigation of the adequacy of various purified diets for the rabbit and of the necessity of preparing diets as low in magnesium as practicable, three diets were used. The composition of these diets is shown in table 1. Since facilities for refrigeration were limited, the rations were generally mixed in lots of 5 kg or less in order to avoid rancidity. In the later series of these experiments, where graded levels of magnesium were required, the diets containing the intermediate levels were prepared by mixing proportionate amounts of the rations containing the extreme levels. More than 70 rabbits were used in these investigations.

## RESULTS AND DISCUSSION

The first experiment was an exploratory one to determine the adequacy of a diet containing a 1:20 liver concentrate as the source of unknown vitamin factors needing further investigation in studying the requirement of the rabbit for magnesium. A group of 6 rabbits was fed diet 10, which contained approximately 6 mg of magnesium per 100 gm (determined by the method of Lindner, '44). A similar group of 6 animals served as a control and received this diet supplemented with 40 mg of magnesium per 100 gm of diet.

After 7 weeks the performances of both groups were not significantly different. One animal in the group receiving the low magnesium diet died during the 4th week, after severe diarrhea, but later results indicated that this could not be specifically attributed to magnesium deprivation. The mean rate of gain for the group fed the low level of magnesium was 165 mg per week with a standard deviation of ± 16 gm, as

TABLE 1

*Composition of experimental diets* [1]

| INGREDIENTS | DIET NUMBER | | |
|---|---|---|---|
| | 10 | 13 | 17 |
| | gm | gm | gm |
| Casein | 20 | 22 | 22 |
| Peanut oil | 10 | 8 | .. |
| Corn oil (Mazola) | .. | .. | 8 |
| Cerelose | 47.9 | 53.9 | 51.9 |
| Cellulose | 12 | 12 | 12 |
| Salts IV-A [2] | 3.6 | 3.6 | 3.6 |
| Wood flour | 2 | .. | .. |
| Liver extract, 1:20 (Wilson and Co.) | 4 | .. | 2 |
| Fortified cod liver oil | 0.5 | 0.5 | 0.5 |

[1] The diets were supplemented with the following vitamins in mg/kg: choline chloride 2000, niacin 200, inositol 100, mixed tocopherols 150, 2-methyl,1-4-naphthoquinone 0.75, pyridoxine 7, thiamine 7, riboflavin 7, and calcium pantothenate 15, with the exception that the two last vitamins were not added to diet no. 10.

[2] The "salts IV-A" mixture was prepared according to the formula for salts IV described by Hegsted et al. ('41), except that the magnesium sulfate was omitted.

compared with 194 gm per week with a standard deviation of ± 61 gm for the control group. Because of the wide variability in the rates of gain of the animals in the control group, the difference here was not considered significant. None of the symptoms characteristic of a magnesium deficiency in other species was observed.

At this stage of the experiment it appeared that the magnesium level of the basal diet was adequate to meet the needs of the rabbit. The males therefore were sacrificed and the

magnesium content of the plasma and whole blood cells determined. A significant difference in these values for the two groups was found. The mean magnesium level of plasma for the males receiving 6 mg magnesium per 100 gm of diet was 1.0 mg per 100 ml, as compared with 1.3 mg per 100 ml for the controls. The mean magnesium contents of the erythrocytes were 6.9 mg and 9.7 mg per 100 ml for the respective dietary treatments.

The 5 females, three in the group receiving the low magnesium diet and two in the control group, were continued on the dietary regimens until all the animals in the deficient group died. By the 10th week a definite decrease in the growth rate of the deficient animals was noted. They began to show alarm and apprehension at the sound of unusual noises. During the 16th week one rabbit suffered a convulsive seizure in which it ran frantically about the cage, and fell on its side in tonic-clonic spasms with eyes dilated and bulging and vasoconstriction of the cutaneous system. When the convulsive seizure ended the animal lay exhausted on its side, but complete recovery occurred in about half an hour (figs. 1–4). Similar seizures were observed in the other deficient animals and the same pattern was noted. One of these rabbits survived 18 observed audiogenic convulsions.

The deficient females survived from 20 to 25 weeks on diet 10, containing 6 mg of magnesium per 100 gm. A loss of weight and a generally unthrifty appearance became evident during the latter part of the experimental period. After the 18th week attempts were made to breed the animals to normal males. Several tries were required in each case before the male was accepted. Death in all three deficient animals followed 8 to 10 days after mating. Post-mortem examination of one animal revealed that ovulation had taken place and was followed by apparent fertilization and fetal implantation, death and resorption.

The two control females continued to grow normally, as evidenced by the fact that from the 8th to the 14th week the average growth was 155 gm per week as compared with simi-

lar females which gained 111 gm per week while fed a commercial stock diet. From this it appeared that 40 mg of magnesium per 100 gm of diet fed under the conditions of this study were adequate to meet the requirements of the growing rabbit.

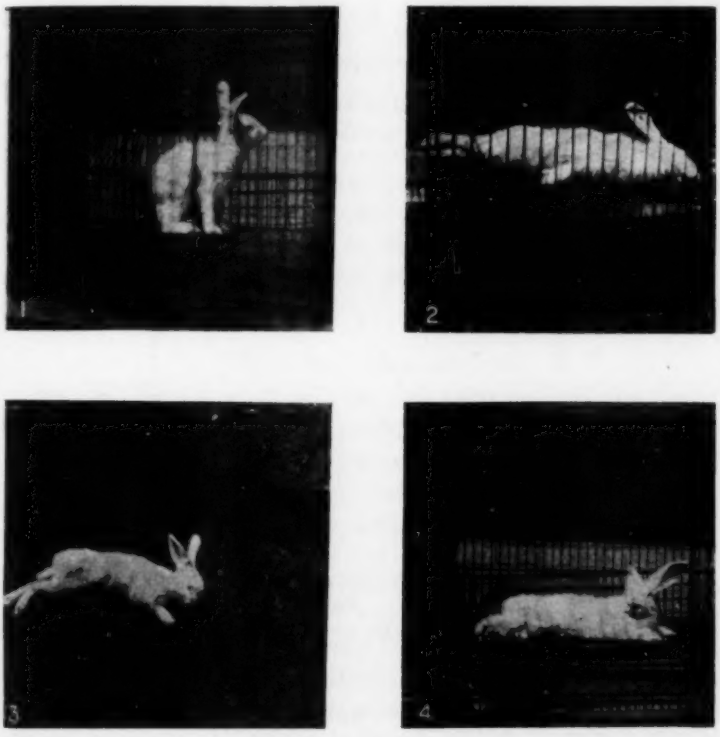

Fig. 1 Magnesium-deficient rabbit apparently normal in appearance before convulsions.

Fig. 2 The same rabbit immediately after the onset of convulsions induced by the sound of an air blast.

Fig. 3 The rabbit immediately following the cessation of convulsions.

Fig. 4 About 5 minutes after the convulsions, the rabbit showing a progressive recovery.

Since the magnesium content of diet 10 appeared to be too high to produce an acute deficiency, a second experiment was designed using diet 13, which contained less than 1 mg of magnesium per 100 gm. Three groups of 5 rabbits each were fed diets containing 40, 6, and less than 1 mg of magnesium in 100 gm of the respective diets. Two of the rabbits in each of the low magnesium groups and one in the group receiving

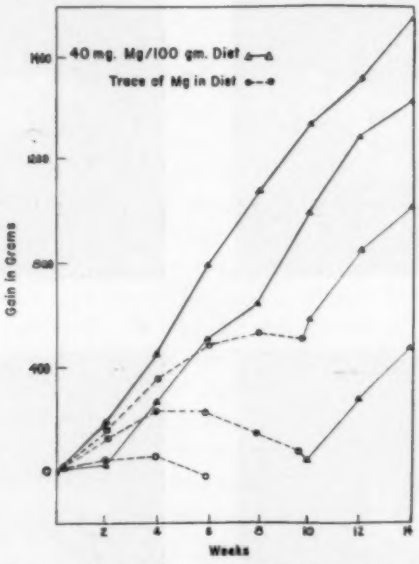


Fig. 5 Effect of magnesium deficiency and the subsequent feeding of magnesium on growth of rabbits.

40 mg of magnesium in 100 gm of diet died after severe diarrhea during the third and 4th weeks.

The symptoms resulting from magnesium deprivation in this experiment seemed to be somewhat more severe than in the preceding one. Retardation of growth was evident by the third week. Convulsions could be induced by the sound of escaping air in the 5th week. A loss of weight and extreme emaciation soon followed the onset of convulsions. By the

9th week all but two animals in the deficient groups were dead. Gross examination of the kidneys of the dead animals revealed a mottled, bloody appearance. Whole blood magnesium of the deficient rabbits after 5 weeks on the experimental diet showed a very significant decrease to a mean value of 3.4 mg magnesium per 100 ml, as compared with 5.5 mg per 100 ml of the blood of animals receiving 40 mg of magnesium per 100 gm of diet. Values as low as 1.8 and 2.4 mg magnesium per 100 ml of whole blood of deficient animals were recorded after the 6th week of the experimental period. Vasodilatation and hyperemia, the symptoms reported to be peculiar to magnesium deficiency in the rat and dog, were not observed in any of the rabbits fed diets low in magnesium.

During the 10th week the two surviving animals on the deficient diets were changed to the diet containing 40 mg of magnesium per 100 gm. There was an immediate response in the rate of growth (fig. 5).

*Quantitative requirements*

The results of these experiments indicate that the quantitative requirement of the rabbit for magnesium under the conditions described is somewhere between 6 and 40 mg per 100 gm of diet. Studies were conducted in which graded levels of magnesium as the sulfate form were fed, to determine the approximate requirement of the rabbit for magnesium and to obtain information on the subacute deficiency. Other investigations on the adequacy of various purified diets (Kunkel, Simpson, Pearson, Olcese and Schweigert, '48) indicate that factors in the liver concentrate are required for maximum growth and general well-being of the growing rabbit. Liver concentrate was therefore included in diet 17, which was the diet used during the subsequent phases of this study.

The criteria used for the adequacy of the magnesium intake were clinical manifestations, growth and the magnesium content of the whole blood.

Ten levels of magnesium, ranging from 7.5 to 47.5 mg per 100 gm of diet were fed. The experimental period was 10

weeks. Blood was drawn at the second, 4th, 6th, 8th and 10th week for determination of the magnesium content. There was a progressive decline to the 8th week in the magnesium content of the blood of rabbits receiving 25.0 mg or less of magnesium per 100 gm of diet: the decline was more severe in the rabbits on the lower levels of magnesium intake. There was no significant difference between the blood magnesium values at the 8th and 10th weeks.

Rabbits fed a diet providing 17.5 mg of magnesium per 100 gm of diet were subject to audiogenic convulsions and other manifestations characteristic of a magnesium deficiency. The growth rate of rabbits receiving diets containing 17.5 mg of

TABLE 2

*Comparison of the effect of magnesium deprivation on the magnesium content of whole blood and plasma of rabbits*

| EXPERIMENTAL PERIOD | ON DIETS WITH MAGNESIUM CONTENT | | | |
|---|---|---|---|---|
| | Less than 20 mg/100 gm | | 37.5 to 47.5 mg/100 gm | |
| | Whole blood | Plasma | Whole blood | Plasma |
| | *(all values are mg magnesium/100 ml blood or plasma)* | | | |
| 10 weeks | 3.15 ± 0.75 (25)[1] | 1.00 ± 0.22 (10) | 4.36 ±0.92 (16) | 1.61 ± 0.38 (8) |

[1] Mean value and standard deviation. Number in parentheses indicates number of animals.

magnesium per 100 gm was 119 gm per week, as compared to gains ranging from 145 to 165 gm with 22.5 mg or more of magnesium.

The average magnesium content of the blood of rabbits fed diets containing 27.5 mg or less of magnesium per 100 gm was less than 4.0 mg per 100 ml, whereas when the intake was 32.5 mg of magnesium per 100 gm of diet the level in the blood was 4.6 mg per 100 ml or higher.

Data on the magnesium content of the whole blood and plasma after 10 weeks on the experimental diets are summarized in table 2. Those rabbits fed 20 mg or less of magnesium per 100 gm of diet have been averaged together and those fed 37.5 mg or more similarly averaged. It is recognized

that rabbits fed levels of magnesium between these levels may have been near the borderline of adequacy of magnesium intake. A diet providing 20 mg or less of magnesium per 100 gm of ration results in levels of magnesium in both the plasma and whole blood that are significantly lower than those on diets providing 37.5 mg or more of magnesium per 100 gm.

The level of calcium in the blood was not affected by the magnesium deprivation. The mean value of serum calcium was 15.5 mg per 100 ml, with no difference among the various groups. Slightly lowered blood cell volume was noted in the more deficient animals.

Since there were no clinical or biochemical manifestations of a magnesium deficiency on diets providing 32.5 mg or more magnesium per 100 gm, the data indicate that the magnesium requirement of the rabbit is between 30 and 40 mg per 100 gm of diet when the magnesium is furnished as the sulfate salt. This level is somewhat higher than that of 20 mg of magnesium per 100 gm of diet which appears to satisfy the requirements of the rat (Kunkel and Pearson, '48).

## SUMMARY

Weanling rabbits fed a diet deficient in magnesium exhibit within a period of three to 6 weeks a syndrome involving hyperexcitability, convulsions, hypomagnesemia, and retardation of growth. The addition of magnesium to the diet of rabbits that have ceased to grow results in a prompt resumption of growth. The vasodilatation characteristic of magnesium deficiency in rats and dogs was not observed in rabbits.

The magnesium content of the blood of rabbits fed a diet adequate to prevent symptoms of a magnesium deficiency was approximately 4.4 mg per 100 ml, while the level for the plasma was about 1.6 mg per 100 ml. On a diet containing 20 mg or less of magnesium per 100 gm of diet the level in the blood shows a progressive decline to about the 8th week of about 3.1 mg per 100 ml for whole blood and 1.0 mg per 100 ml for plasma.

The magnesium requirement of the rabbit appears to be between 30 and 40 mg per 100 gm of diet.

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# VOLUNTARY CALORIC INTAKE OF THE GROWING RAT

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TWO FIGURES

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It is a common observation in laboratories engaged in rat growth studies that animals growing poorly ingest less food. In repletion studies such as those described by Cannon and co-workers ('44) animals showing poor weight recoveries likewise eat smaller quantities of food.

The fact that animals growing poorly do ingest less food has prompted numerous modifications in technics for the study of ration quality wherein attempts are made by one means or another to equalize food intakes. A strong positive correlation exists between weight gain and food intake. For example, Harte and Travers ('46) have analyzed data for 279 male rats fed diets of constant composition except for protein quality and found a correlation coefficient of + 0.82 between these variables, while similar data on 61 rats fed a diet containing casein showed a correlation coefficient of + 0.67. Hegsted and Worcester ('47) studied similar data after logarithmic transformation of the variables and reported a correlation coefficient of + 0.83 between the transformed variables.

Examination of data in the literature and preliminary observations suggested the existence of a constant relationship between the voluntary caloric intake of growing rats fed ad

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libitum and the size they attained. The experiments to be described in this report were carried out in an attempt to establish this relationship for the growing rat.

In part of the experimental work the design was such that analyses of variance enabled the drawing of collateral conclusions on the roles of sex and litter as well. It is worthwhile to emphasize, as Addis and associates ('48) have done, how valuable the application of modern small-sample statistical theory to experimental data can be. By means of these technics, various aspects of the data of a single well-designed experiment may be evaluated and hypotheses tested without setting up a series of individual comparisons. In this way the efficiency of experimentation is markedly enhanced and the same amount of laboratory work may be made to yield substantially more information.

## EXPERIMENTAL

Two series of tests were carried out. In the first, 7 litters born over a period of about one month by breeders from our own colony (Sherman strain albino rats, originally obtained from Rockland Farms) were used. The litters totalled 17 males and 21 females. These animals were weaned 21 days after birth and fed semi-synthetic Ration A (table 1) ad libitum. After 9 weeks all animals except those from one litter were transferred to Ration B (table 1), on which they were maintained for an additional three weeks. The ration compositions were designed to make the ration of protein calories to total calories the same.

In the second series of experiments weanling males, purchased to a weight specification, were used. The general technic and ration composition have been described by Harte et al. ('47), except that biotin was included in all rations at a level of 0.1 mg per kilogram and a series of different proteins [3] were substituted for the casein used in the experiments

[3] Most of the proteins used in this part of the study were provided by the Bureau of Biological Research of Rutgers University as part of its program for the collaborative study of protein assay methods. The results of growth tests with these proteins will be reported elsewhere.

cited. Appropriate adjustments in the cornstarch content of these rations made them isonitrogenous and they were assumed to be isocaloric.

Caloric intakes were calculated from food intakes and the caloric values of the rations; the latter were estimated by assuming that protein and carbohydrate yield 4 Cal. per gram and fat, 9 Cal. per gram. While calorimetric determinations might have been preferred, these assumptions were considered sufficiently accurate for the present purposes, particularly in the light of the uncertainties involved in the estimation of surface areas.

TABLE 1

*Ration composition* [1]

| COMPONENT | RATION | | COMPONENT | RATION | |
|---|---|---|---|---|---|
| | A | B | | A | B |
| | % | % | | % | % |
| Dried skim milk | 8.38 | 7.25 | Cornstarch | 50.3 | 57.0 |
| Casein | 13.4 | 11.6 | Lard | 9.0 | 1.0 |
| Lactalbumin | 3.35 | 2.9 | Ruffex | ... | 5.0 |
| Malt sugar | 8.37 | 7.25 | | | |

[1] In addition, each kg of ration contained salts, 40 gm; wheat germ oil, 10 gm; cod liver oil, 10 gm; liver extract concentrate (Wilson's Liver Extract Concentrate, 1:20, was used) 10 gm; choline chloride, 1.8 gm; thiamine hydrochloride, 5 mg; riboflavin, 8 mg; pyridoxine, 6 mg; calcium pantothenate, 13 mg; and niacin, 13 mg.

Computed Calories per gram: 4.47 for ration A, 3.84 for ration B.

The salt mixture used had the same composition as that described by Phillips and Hart ('35) but was modified by the addition to their formula of 0.2 gm of potassium alum and 1 gm of sodium fluoride.

In the handling of the data accumulated during the experimental periods, surface areas of the individual animals were estimated from the formula $A = 11.36\ W^{2/3}$, reported by Carman and Mitchell ('26). Average areas for each animal during any given week were obtained by averaging the areas corresponding to the weights at the beginning of the week and at the end of the week.

## RESULTS

The average growth of the animals in the 7 litters used in the first experiment, as evidenced by changed body weight, is shown in figure 1. This growth was quite satisfactory, averaging 4.8 gm per day for the males and 2.8 gm per day for the females during the 63 days on Ration A. These data fit straight lines in the log weight-reciprocal age coordinate system proposed by Zucker and Zucker ('42), with slopes differing only slightly from those described as optimum. For

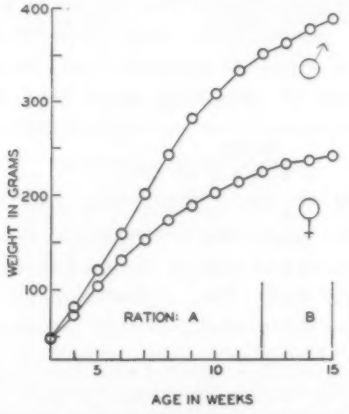


Fig. 1 Growth curves for male and female rats fed ration A through the 12th week of life and ration B thereafter. Of the 17 males and 21 females fed ration A, 15 males and 17 females were continued on ration B.

females the slope is 2.74 compared to Zucker's 2.83; for males, 3.54 compared to Zucker's 3.71. Evidence of the excellence of fit to linearity is found in the values of the rectilinear correlation coefficients between reciprocal age and log weight, which are —0.9984 for females and —0.9958 for males.

While analyses of variance indicated very clearly that the 9-week gains for both males and females had substantially smaller variance within litters than between litters, this significant difference, which would favor the use of litter mates, disappeared when differences in initial weight were taken

into consideration by application of the methods of covariance. Differences in initial weight, sex being the same, had a greater influence on weight gain than whether or not the animals were litter mates.

In figure 2 are plotted, for males and females separately, the voluntary calorie intakes of these animals calculated as Calories per square decimeter per day. The data consistently indicate a higher value for the 5th week of life than for the 4th, but after the 5th week a steady decrease was observed, the intake approaching as an approximate limit a value of between 12.5 and 13 Cal. per square decimeter per day for the animals of both sexes.

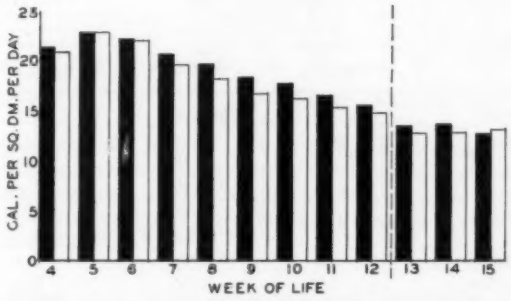


Fig. 2 Bar chart giving mean voluntary caloric intake in Calories per square decimeter of body surface per day for weekly intervals. Solid bars represent males; open bars, females. Values to the left of the dashed line were obtained with ration A; to the right, with ration B. Ration A was fed to 17 males and 21 females; ration B to 15 males and 17 females.

A complete analysis of variance of these data, summarized in table 2, showed that the observed differences from week to week during both the first 9 weeks on the ration containing 4.47 Cal. per gram, and the last three weeks on the ration containing 3.84 Cal. per gram, were statisically highly significant. On the other hand, during the first 9 weeks the difference in caloric intake between the sexes was highly significant while during the last three weeks it was not significant. Similarly, for both males and females during the first 9 weeks

differences between litters were significant as compared to differences within litters, while during the last three weeks the inter-litter differences were not significant for either sex.

The second series of experiments were carried out with rations practically identical with each other except for protein source. At the end of the 5-week feeding period the animal weights reflected the differences in protein quality by

TABLE 2

*Ad libitum calorie consumption — analysis of variance*

| SOURCE OF VARIATION | RATION WITH 4.47 CAL. /GM FED 1 TO 9 WEEKS INCLUSIVE | | RATION WITH 3.84 CAL. /GM FED 10 TO 12 WEEKS INCLUSIVE | |
|---|---|---|---|---|
| | Degrees of freedom | Mean square | Degrees of freedom | Mean square |
| Between weeks | 8 | 296.349 [1] | 2 | 8.061 [2] |
| Within weeks | 333 | 2.412 | 93 | 1.385 |
| Individuals | 37 | 9.757 | 31 | 2.073 |
| Sexes | 1 | 79.190 [2] | 1 | 3.508 |
| Within sexes | 36 | 7.828 | 30 | 2.025 |
| Males | 16 | 6.959 | 14 | 1.231 |
| Inter-litter | 6 | 13.069 [3] | 5 | 0.884 |
| Intra-litter | 10 | 3.294 | 9 | 1.424 |
| Females | 20 | 8.524 | 16 | 2.720 |
| Inter-litter | 6 | 20.826 [3] | 5 | 4.827 |
| Intra-litter | 14 | 3.252 | 11 | 1.763 |
| Discrepancy | 296 | 1.494 | 62 | 1.040 |

[1] F value exceeds that tabulated for $p = 0.001$.
[2] F value exceeds that tabulated for $p = 0.01$.
[3] F value exceeds that tabulated for $p = 0.05$.

ranging from 62 to 169 gm. Voluntary caloric intake in Calories per square decimeter per day were computed from the data as before. The analysis of variance of these data indicated that the possibility that the differences in unit caloric intake observed with time might be due to chance is extremely small (p less than 0.001). Far more striking is the observation that the differences in caloric intake with different rations

are not statistically significant (p greater than 0.20). In table 3 the averages for the unit caloric intakes of the animals (all males) receiving these different rations are given, as well as averages of the observed weight ranges and gains.

TABLE 3

*Weight gains and average caloric intakes of growing rats on rations containing proteins of different qualities (N × 6.25 = 10%)*

| PROTEIN SOURCE | BODY WEIGHT | | CALORIC INTAKE DURING WEEK | | | | | NO. OF RATS |
|---|---|---|---|---|---|---|---|---|
| | Initial | Gain [1] | 1 | 2 | 3 | 4 | 5 | |
| | *gm* | *gm* | *Cal./day/dm² of body surface* | | | | | |
| Casein I | 56.6 ± 1.8 [2] | 77.6 ± 3.9 | 21.2 | 18.4 | 18.3 | 17.5 | 16.5 | 10 |
| Casein II | 56.7 ± 1.5 | 71.3 ± 2.6 | 20.4 | 17.6 | 16.6 | 16.9 | 17.7 | 9 |
| Beef | 54.0 ± 1.6 | 85.7 ± 6.3 | 20.2 | 19.2 | 18.7 | 18.1 | 17.1 | 9 |
| Whole egg | 52.6 ± 2.1 | 105.4 ± 4.1 | 20.9 | 19.9 | 17.9 | 17.1 | 16.8 | 9 |
| Peanut flour | 55.3 ± 1.7 | 31.3 ± 3.0 | 21.0 | 19.4 | 17.3 | 17.9 [3] | 17.9 [3] | 10 |
| Wheat gluten | 56.9 ± 1.8 | 14.4 ± 1.8 | 19.3 | 18.4 | 17.7 | 17.8 | 16.5 | 9 |
| Weighted mean | | | 20.5 | 18.8 | 17.8 | 17.6 | 17.1 | |

[1] Weight gains are those recorded after ad libitum feeding for 5 weeks.

[2] Indicated limits represent standard errors of the reported means.

[3] During the latter part of the 4th week and the beginning of the 5th week these animals received protein at a somewhat lower level due to an error in making up one batch of this ration.

When these data were calculated on the basis of Calories per 100 gram of body weight per day, the analysis of variance showed the differences between rations to be very highly significant. This recalls the importance of the evidence linking metabolic phenomena with body surface, as reviewed by Brody ('45). Data were also available, but are not reported here, on a similar ration containing egg white. It appeared that animals fed this ration consumed significantly fewer Calories per square decimeter per day, although they grew very well. Additional study will be required to verify this observation.

## DISCUSSION

The voluntary eating habits of the growing rat as elucidated by these experiments show surprising uniformity from ani-

mal to animal. That young males will voluntarily ingest food equivalent to more energy per unit of surface area than females of the same age might be expected. In humans, boys tend to have a higher basal metabolism than girls and this difference between the sexes persists well into maturity but seems to disappear after about 60 years of age. The analogous situation with respect to rats has been studied by Mitchell and Carman ('26).

The occurrence of a maximum in voluntary unit caloric intake during the 5th week of life in the growing rat finds its parallel in the presence of a maximum in the relation between resting metabolism and age (Brody, '45). This latter maximum seems to occur somewhat later, coinciding roughly with an age of 40 days. In some measure, then, the changing voluntary caloric intake reflects, or is reflected by, a changing unit resting metabolic rate.

The energy requirements of the mature rat have been estimated by various workers. Benditt et al. ('48) have shown that the utilization of a constant quantity of protein is restricted when the caloric intake falls below about 12.4 Cal. per square decimeter per day in the protein-depleted rat. Bosshardt et al. ('46) found that the critical caloric value for the growing rat is approximately 12.5 Cal. per square decimeter per day, averaged over a 42-day period. In the light of the changing unit demand and the nature of the relation between weight and area, this period may be too long to yield an entirely valid average. During the most active period of growth after weaning our rats voluntarily ingested food equivalent to almost twice that number of Calories, but their unit consumption tended to decrease quite regularly and apparently to stabilize in the vicinity of 12.5 to 13 Cal. during the 14th and 15th weeks of life.

No correction was made to accord with the viewpoint of Cherkin ('48) that dietary nitrogen utilized exclusively for protein functions should not be included in estimating the energy content of the diet. Since some of the dietary fat and carbohydrate may be retained as glycogen or tissue fat, cor-

rection in the estimation of their contributions to the total Calories would also be required. Whether dietary calories from any source are released as heat promptly after ingestion or are stored to become potential Calories does not affect the over-all bookkeeping necessary to establish the status of caloric balance. Stored foodstuffs take the form of potential Calories, rather than realized Calories, and these considerations make Cherkin's argument misleading except in a highly specialized, short-term sense.

Of course if appetite is a factor there is no reason to suppose that any relation need exist between the animal's caloric requirements and the number of calories which it will voluntarily ingest. There is some indication that this may represent the state of affairs when diet composition is markedly varied. Preliminary observations on adult rats receiving protein-free rations indicate that these animals will voluntarily ingest fewer calories than similar animals offered rations containing 5 or 10% casein. Also, when the composition of the diet is changed — e.g., from 4% fat to 20% fat — the voluntary caloric intake rises enormously and persists at a high level for as long as two weeks before dropping to the previously attained plateau value. The situation does not seem to be simple because to some extent, at least, the animal eats to satiety. For a mature animal maintained on a given diet satiation might require, let us say, 18 gm of ration daily. When such a diet is replaced by one of higher caloric content, approximately the same bulk will be required initially to provide the same degree of satiety and as a result the caloric intake will rise until metabolic adjustments take place.

Particular interest attaches to the apparent uniformity of voluntary caloric intake on rations differing only in their protein quality. Under these conditions, it would appear that the primary purpose in eating is to satisfy energy requirements and that their satisfaction is a function of the age and sex (which determine the unit requirement) and the size of the animal. Animals of the same age and sex starting at the same size will initially ingest equivalent calories, both in

totality and per unit of surface area. If as an accidental corollary to the ingestion of these calories some of the animals become larger, they will voluntarily ingest more calories, simply because there are a larger number of units of surface area seeking caloric satisfaction. It would appear, then, that weight gain is merely incidental to the satisfaction of energy demands. So long as the animals are receiving rations of essentially constant composition, they will ingest just sufficient food to satisfy their caloric requirements per unit of surface area. Differences in food consumption observed with different rations will, under these conditions, merely reflect differences in animal size.

In carefully conducted studies with adult dogs, Cowgill ('28) has shown that these animals voluntarily regulate their food intakes to satisfy their energy requirements and, other things being equal, do so in obedience to the so-called surface area law. The present findings with the growing rat are in full accord with Cowgill's speculative extra polation of his findings to the feeding of infants.

These considerations offer an explanation for the observation previously noted that if the protein quality is poor the animals ingest less food. An unsuitable assortment of amino acids, reflecting deficiencies in the protein's pattern or lack of digestibility, does not permit the formation of new tissue. Failure of size to increase has as its consequence the reduction of total caloric intake.

With respect to rats and proteins, one may well say "If it's a poor protein, they won't eat well," but it cannot be emphasized too strongly that no evidence has been presented for the reciprocal statement, "If they won't eat well, it's a poor protein." Strong temptation to invert the first statement into the second has become the basis of reckless inferences for which no justification exists.

On cursory examination it would appear that the 9-week weight gains are more uniform for animals of the same sex and litter than for animals of the same sex chosen randomly with respect to litter. This conclusion, however, does not take

into consideration variations in initial weights in litter mates of the same sex. When appropriate adjustments are made for the divergence in weaning weights of litter mates of the same sex, then there is just as much uniformity among animals of the same sex, age and weaning weight without regard to litters as is found in animals of the same sex and litter. Our data definitely suggest the possibility that the former criteria of selection might provide even more uniform animals. This is in agreement with the implicit hypothesis of Zucker's growth equation (Zucker and Zucker, '42), which suggests that the growth curve of an optimally-fed animal is uniquely determined by its weaning weight. For any given strain the slope term of Zucker's equation is given as a constant not subject to inter-litter variation.

## SUMMARY

1. The voluntary food consumptions of 21 female and 17 male rats from 7 litters were studied during the first 12 weeks after weaning.

2. During the second week after weaning the voluntary caloric intake per unit of surface area increases over that observed in the first week; and thereafter it decreases, leveling off at a value between 12.5 and 13 Cal. per square decimeter per day.

3. To some extent this equilibrium value may be related to the proximate composition of the ration.

4. Given rations of the same proximate composition but differing in their ability to promote growth as a consequence of differences in protein quality, the voluntary caloric intake per unit of surface area per day was constant during 5-week growth studies, regardless of the quality of the protein component, when the protein source was casein, beef, whole egg, peanut flour or wheat gluten.

5. This apparent constancy of unit caloric intake emphasizes that the primary urge in the ingestion of food is the satisfaction of energy requirements and that, other things

being equal, if growth ensues it is, within limits, an accident of the make-up of the ration.

6. The data indicate that animals will be more uniform in their growth responses if they are selected on the basis of sex and weight at a given age than if the latter factor is ignored in favor of the criterion that they be litter mates.

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# THE EFFECT OF THE COMPLEX-FORMING AMORPHOUS SODIUM PHOSPHATES ON THE CALCIUM RETENTION OF RATS[1]

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ONE FIGURE

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## INTRODUCTION

The demonstration that tartrates (Hamilton and Schwartz, '33) and citrates (Hamilton and Dewar, '37; Shohl, '37) prevent rickets when added to a rickets-producing diet has resulted in several investigations designed to explain this action. Originally it was suggested that acid-base factors were largely responsible (Hamilton and Schwartz, '33). This was confirmed in part by the subsequent work of Shohl ('37), who nevertheless showed that this hypothesis was inadequate, as did Hamilton and Dewar ('37). The former stated that although "the acid reaction and alkali ash factors are necessary," an investigation of the complex-forming properties of the tartrates and citrates would be of value. The latter investigators suggested that the citrates and tartrates may lead to a decrease in the availability of calcium, either by complex formation or by the formation of insoluble calcium salts other than phosphate, thereby increasing the probability of the absorption of phosphates and in this manner preventing rickets. They showed too that the salts of citric and tartaric acids are

[1] Taken from a thesis submitted by M. K. Borenstein in partial fulfillment of the requirements for the degree of Master of Arts, University of South Dakota, 1947.

more effective in rickets prevention than the free acids. The work of Hathaway and Meyer ('39) indicates that the potassium salts are more effective than the sodium salts. Day ('40) suggests that the "beneficial effect of citrates is mediated through the formation of a calcium complex which interferes less, if at all, with the utilization of phosphorus in phytin."

In view of the strong probability that complex formation may play an important part in the effects of the citrates and tartrates, it seemed of interest to determine the effects on calcium metabolism of other calcium complex-forming materials. Of the complex formers now known, amorphous sodium phosphate is probably the most effective. Consequently, the calcium and phosphorus retentions of rats on the Steenbock-Black ('25) diet to which amorphous sodium phosphate was added were determined, as well as similar retentions of rats on the same basal diet to which was added a crystalline, soluble, non-complex-forming phosphate of approximately the same composition.

## EXPERIMENTAL METHODS

### *Rats and metabolism cages*

Male rats (Sprague-Dawley, Inc., Madison, Wis.) between the ages of 24 and 28 days were used. The metabolism cages, cylindrical and made of wire mesh, were similar in principle to those described by Brooke and Smith ('33).

### *Collection of samples*

The collection of urine and feces was carried out essentially as described by Brooke and Smith ('33), with the following modifications: (1) The acetic acid washed filter papers used for collecting the urine were pressed dry on a letter press rather than sucked dry on a Buchner funnel; (2) for extraction, the filter papers were macerated in a Waring-type blender, using the cage washings as the fluid medium. The slurry of macerated filter paper and cage washings was al-

lowed to settle overnight before filtration and washing by suction. The filtrates were evaporated to a convenient volume, transferred quantitatively to volumetric flasks and made up to volume; aliquots were used for calcium and phosphorus determinations.

The efficiency of the extraction procedure was checked by further extraction of the filter paper residue with 2 N HCl. No calcium was found in the hydrochloric acid extract, and phosphorus to the extent of only 0.1% of the total in the urine sample was recovered.[2]

Occasionally some spilled dried food remained on a filter paper used for the collection of urine. The amount in general was small and would not influence the final retention figures.

*Preparation of samples*

For the determination of calcium the urine samples required no ashing. Dry ashing of the urine for the determination of phosphorus proved to be less satisfactory than a wet ashing procedure. The desired aliquot was dried in a pyrex digestion tube in a water bath. The residue was heated with 1 ml of concentrated sulfuric acid until charring occurred, then the cautious, drop by drop addition of 2 to 5 drops of perchloric acid while heating was continued served to complete the digestion. During heating the tubes were covered with a cone-shaped piece of pyrex glass about one inch high, with its apex downward and offset so as to return any condensate along the wall of the tube. The base of the cone, which rested on the lip of the digestion tube, was flared unevenly in order to minimize the effects of "bumping."

The feces were ashed by the method of Berggren ('32). The ashed samples were taken up with 1 ml of distilled water and 1 ml of concentrated hydrochloric acid, heated on the water bath for 30 minutes (water and acid being replaced as they evaporated), transferred quantitatively to volumetric flasks, and aliquots taken for analysis.

[2] No correction was made for this in computing the final results, since the amount is insignificant.

### *Analytical methods*

Calcium was precipitated as the oxalate from aliquots of both urine extract and feces ash by the method of Van Slyke and Sendroy ('29), washed, dissolved in 1 N sulfuric acid, and titrated with 0.01 N potassium permanganate using the modified Tisdall-Kramer method as described by Clark and Collip ('25). The determinations were checked from time to time by the use of solutions of known calcium content.

Phosphorus was determined on the ashed urine and feces by the method of Fiske and Subbarow ('25) using a Coleman spectrophotometer. Phosphates were run in groups of 20 to 40 individual determinations and a standard curve was made for each group. Check determinations were run periodically, using the volumetric method of Lundell and Hoffman ('24).

All analyses were run in duplicate, and in any instance where duplicate determinations of either calcium or phosphorus failed to agree to within 5 parts per 100, both determinations were repeated.

### *Diets*

The Steenbock-Black ('25) rickets-producing diet was used as the basal diet. Amorphous sodium phosphate with a ratio of $Na_2O$ to $P_2O_5$ of about 1.1 to 1 was used as received.[3] Crystalline soluble sodium phosphate with approximately the same ratio of $Na_2O$ to $P_2O_5$ was purified before use by recrystallization from hot water. The calcium and phosphorus content of each diet was determined by the methods previously indicated. Samples for analysis were obtained in each case by quartering from 330 gm of the well mixed diet. The calcium and phosphorus contents of the diets used are shown in table 1.

As will be noted, there is some slight variation in diet compositions. This is probably due to the fact that each diet

[3] The phosphates used in this work were obtained through the courtesy of Calgon, Inc., Pittsburgh, Pa. The amorphous material is commonly known as sodium "hexametaphosphate." The soluble crystalline substance is called sodium "trimetaphosphate." To save space the former will be called Am.P., the latter Cry.P., in this paper.

was mixed separately and that during the course of this work the supply of some of the dietary components was replenished.

### *General procedure*

Each animal was kept throughout the experiment in an individual cage. Cages were cleaned and food and water renewed three times weekly. Once a week each cage was washed down thoroughly with hot distilled water and 2 N acetic acid. The excreta for each rat were combined for a one-week period. Each experiment lasted for 4 weeks. Rats were handled in groups

TABLE 1

*Calcium and phosphorus contents of diets used*

| DIET | | Ca | P | Ca/P |
|---|---|---|---|---|
| | | % | % | |
| 1. Steenbock-Black | (a) | 1.30 | 0.33 | 3.94 |
| (Five different | (b) | 1.28 | 0.35 | 3.66 |
| mixes were used | (c) | 1.19 | 0.27 | 4.41 |
| in the course of | (d) | 1.17 | 0.26 | 4.50 |
| this work.) | (e) | 1.15 | 0.25 | 4.60 |
| 2. Steenbock-Black + 7% Am.P.[1] | | 1.19 | 2.54 | 0.47 |
| 3. Steenbock-Black + 7% Cry.P.[2] | | 1.05 | 2.13 | 0.49 |
| 4. Steenbock-Black + 3½% Am.P. | | 1.16 | 1.34 | 0.87 |
| 5. Steenbock-Black + 3½% Cry.P. | | 1.14 | 1.28 | 0.89 |

[1] Amorphous material commonly known as sodium "hexametaphosphate."
[2] Crystalline substance called sodium "trimetaphosphate."

of 12. Six animals were maintained on the phosphate-containing diet under experiment, three were maintained on the same diet supplemented with 30 I.U. of vitamin D for every 5 gm of food consumed, and three were kept on the unaltered Steenbock-Black diet. A preliminary experiment with 6 rats on the basal Steenbock-Black diet was carried out as a check on procedure before any work with the phosphates was begun.

When the 4-week experimental period had elapsed, all rats were killed and x-rayed to determine the presence or absence of rickets.

## RESULTS AND DISCUSSION

A summary of the results of the analyses carried out during the course of this work is given in table 2.

A study of the data obtained based on one of the usual tests for significance (Snedecor, '40) indicates the following:

1. The amorphous sodium phosphate used, when added to the Steenbock-Black diet in the amount of 7%, results in greater calcium retention than that observed when the same amount of crystalline phosphate of approximately the same composition is used, although the Ca to P ratios for the diets are almost equal ($t = 6.1$).

2. When the phosphates are added to the diets in amounts of 3½% of the total weight, differences of little or no significance are observed between the calcium retention of the rats on one diet and that of the rats on the other.

3. The ingestion of vitamin D by the rats on the diets containing either 7% or 3½% of the phosphates did not alter the results significantly from those obtained without the added vitamin.

Previous work with the complex phosphates has not been extensive and in general is not strictly comparable to the work herein reported. Shelling in 1932 found that parathyroidectomized rats fed on a low-calcium, high-phosphorus diet developed tetany, while those maintained on a high-calcium, low-phosphorus diet did not show symptoms of this disorder. In addition, he noted that the rats had a tendency to refuse the tetany producing diet, lose weight, and become cachectic. In this connection it was observed that the high-phosphorus diets would induce the tetany symptoms in the rat when the phosphorus was in the form of the ortho- or pyrophosphate; when it was in the form of the metaphosphate or hypophosphate it was ineffective in producing tetany. On the basis of these observations Shelling assumed that the metaphosphate and the hypophosphate are "either inert biologically or excreted unchanged."

The data presented in the present paper indicate that Am.P. (amorphous sodium hexametaphosphate) and Cry.P. (soluble

TABLE 2

*Ca and P retentions for each of the diets used*[1]

| DIET | NO. RATS | INTAKE | | EXCRETION | | | | | | RETENTION | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Ca | | | P | | | Ca | | | P | | |
| | | Ca | P | Urine | Feces | Total | Urine | Feces | Total | Absolute | % | Range | Absolute | % | Range |
| | | | | | | | | | | | | % | | | % |
| Steenbock-Black + 7% Am.P.[2] | 6 | 1762 | 3754 | 129 | 1034 | 1163 | 1047 | 1458 | 2505 | 599 | 33.9 | 26.6–37.2 | 1249 | 33.3 | 22.4–43.4 |
| Steenbock-Black + 7% Cry.P.[3] | 6 | 1563 | 3164 | 229 | 1118 | 1347 | 1116 | 1334 | 2450 | 216 | 13.8 | 6.5–23.1 | 714 | 22.5 | 18.4–27.0 |
| Steenbock-Black + 7% Am.P. + vit. D | 3 | 1881 | 4003 | 184 | 980 | 1164 | 1273 | 1418 | 2691 | 717 | 38.2 | 36.8–40.0 | 1312 | 32.8 | 31.7–34.1 |
| Steenbock-Black + 7% Cry.P. + vit. D | 3 | 1611 | 3261 | 211 | 1100 | 1311 | 1062 | 1354 | 2416 | 300 | 18.6 | 17.4–19.5 | 845 | 25.9 | 21.4–28.8 |
| Steenbock-Black + 3½% Am.P. | 6 | 1533 | 1776 | 113 | 1052 | 1165 | 361 | 948 | 1309 | 368 | 24.0 | 21.1–31.9 | 467 | 26.3 | 19.4–33.1 |
| Steenbock-Black + 3½% Cry.P. | 6 | 1421 | 1585 | 166 | 984 | 1150 | 272 | 874 | 1146 | 271 | 19.0 | 13.1–25.8 | 439 | 27.7 | 16.8–35.2 |
| Steenbock-Black + 3½% Am.P. + vit. D | 3 | 1731 | 2003 | 169 | 982 | 1151 | 451 | 952 | 1403 | 580 | 33.5 | 29.2–37.2 | 600 | 30.0 | 26.4–33.4 |
| Steenbock-Black + 3½% Cry.P. + vit. D | 3 | 1616 | 1819 | 250 | 971 | 1221 | 396 | 936 | 1332 | 395 | 24.4 | 17.7–29.3 | 487 | 26.8 | 21.8–29.4 |

[1] All values are 4-week totals in terms of mg/rat, except as otherwise noted.
[2] Amorphous material commonly known as sodium "hexametaphosphate."
[3] Crystalline substance called sodium "trimetaphosphate."

crystalline sodium trimetaphosphate) are not inert, or at least that they are absorbed from the diet as evidenced by the large increases in urinary phosphorus when they are ingested; that Am.P. is effective biologically is shown by the increase in the calcium retention of rats to which it is fed. Furthermore, fresh samples of either material show only traces of orthophosphate, whereas fresh urine samples from rats fed diets containing either of these substances showed large amounts of orthophosphate, indicating that they are hydrolyzed in the body. Thus, it is probable that the metaphosphate to which Shelling refers was neither Am.P. nor Cry.P. but rather a commercially available insoluble crystalline material which may, in fact, because of its relatively poor solubility, be inert.

Similarly, Fraser and his co-workers ('46) report that the insoluble sodium metaphosphate is far less available when fed to rats on a phosphorus-deficient diet than the soluble orthophosphate. They also found that the orthophosphate is more effective in stimulating the storing of both phosphorus and calcium, particularly when oxalate or ferric ions are present in the diet. This is not too surprising in view of the relative solubilities of the particular phosphates used. These authors also report an experiment involving retentions of calcium and phosphorus on a low-phosphorus diet in the presence of ferric citrate, where a loss of calcium is noted which is obviously due to a loss of soft tissue. Since the diets they used differ markedly in phosphate content from those herein reported, comparison of their results with those obtained in this work is not possible.

In another report involving the addition of Am.P. to the diet of rats, Schwartz and his co-workers ('40) observed the effect of Am.P. treated milk on a group of rats and found that the daily urinary output of calcium and the net calcium retention were increased while the daily fecal calcium was decreased.

Murray ('40), investigating the pharmacological action of the metaphosphates, found that the introduction of Am.P. into the blood stream would result in symptoms such as might

be expected with the removal of calcium ions from the blood. He also showed that when calcium and the amorphous metaphosphate were mixed prior to injection into the blood stream the above effects were not elicited. No symptoms of tetany were observed in the course of this study. It would appear, therefore, that Am.P. is normally absorbed with sufficient calcium to nullify its tetany-producing properties or that it is hydrolyzed during the course of absorption to orthophosphate and after absorption behaves as does the latter.

From these reports and the data obtained in the present investigation it is obvious that the soluble complex phosphates, at least insofar as Am.P. and Cry.P. are concerned, are absorbed and excreted and are physiologically active, in that they appear to exhibit *in vivo* the same properties that they exhibit *in vitro*.

Am.P., which forms complexes with calcium *in vitro*, does increase calcium retention significantly when added to the Steenbock-Black diet in 7% quantities. Cry.P., which does not form complexes *in vitro*, does not have a corresponding effect when added in the same amount. Since both of these materials are neutral in character, the increased calcium retention when Am.P. is used can at present be explained only in terms of its complex-forming properties. Thus in the digestive tract the presence of a sufficient quantity of Am.P. probably results in the formation of a soluble calcium-metaphosphate complex ion,[4] which is absorbed. Any excess phosphate and calcium are excreted through the kidneys except for the probably insignificant amount excreted through the intestines, the rest being retained.

In the presence of Cry.P., which does not react with calcium, calcium and Cry.P. are absorbed independently. After or during absorption the Cry.P. is hydrolyzed to orthophosphate, the excess excreted, and the remainder retained with calcium.

In the presence of water both substances are hydrolyzed finally to the orthophosphate with a velocity determined by

[4] The chemistry of these materials is discussed in greater detail by Schwartz et al. ('40).

concentration, pH, temperature and other factors. It is not likely that such hydrolysis takes place to any appreciable extent in the digestive tract since, if it did, no great difference in results between using Am.P. and Cry.P. would have been observed. Furthermore it is not likely, for the reasons previously given, that these substances pass into the blood unchanged. Thus it would appear that they are both absorbed as complex phosphates, with or without calcium as the case may be, and that during or after this process are hydrolyzed to orthophosphate, the excess of which is subsequently excreted.

When only 3½% of the phosphates is added to the diets, no significant difference in calcium retention is observed. A possible explanation may lie in the chemical properties of Am.P. It is well known that if Am.P. is carefully added to a calcium solution a gelatinous precipitate forms, possibly $[Ca(PO_3)_2]_n$. This is relatively insoluble but is dissolved by an excess of Am.P. When 3½% of the latter is added to the diet it is conceivable, therefore, that $[Ca(PO_3)_2]_n$ is the predominant result, rather than the soluble complex ion. Since the former is relatively insoluble, no increase in absorption would necessarily result. This would appear to be the case if it is assumed that all calcium not appearing in the feces is absorbed. As might be expected, the addition of vitamin D to the phosphorus-rich diets did not alter the results significantly.

No rickets could be detected by x-ray examination of the rats on any of the phosphate supplemented diets. The change of the calcium-phosphorus ratio by the addition of the phosphates obviously renders the Steenbock-Black diet ineffective in the production of rickets. All control rats on the basal diet without added phosphates did develop rickets.

A severe diarrhea was noted within a few days after the rats were placed on the diets containing both the amorphous and crystalline sodium metaphosphates at a level of 7%. The high concentration of salts, with the probable concomitant dehydration of the gastrointestinal tract resulting in an increase of fluid in the alimentary tract, seems to be the under-

lying cause. Since no diarrhea was observed when the animals were maintained on the diet containing 3½% added phosphates, it can safely be surmised that the phosphate itself does not have a specific effect on the production of diarrhea. In addition to the symptoms of diarrhea, the animals on the diet containing 7% of phosphate excreted urines which had a peculiar reddish tinge. Tests for occult blood proved negative. No analyses for heme compounds were made.

Pathological examination of the visceral organs of the rats in the experiment using 7% amorphous metaphosphate failed to show any abnormalities, either in microscopic sections[5] of the visceral organs or on macroscopic examination. A cloudy swelling was observed in the tubular epithelium of the kidney, which cannot be considered pathological. Diarrhea in rats when the amorphous sodium metaphosphate amounted to 10% of the diet has been reported (Schwartz et al., '40). The results of pathological examination in that study were essentially the same as those observed in the present investigation. The action of the amorphous metaphosphate seems to be similar in effect to that of any mineral cathartic.

As a check on the experimental methods used, groups of rachitic control rats on the basal Steenbock-Black diet were studied during each expermient. Data were obtained from a total of 18 rats and a summary of the observations made is presented in table 3. The retentions found are of the same order of magnitude as those reported by others, although there are some minor differences. While there was considerable variation in the absolute quantities of calcium and phosphorus retained, the relation between calcium and phosphorus retentions was quite good and all 18 values for the 4-week retentions fell quite close to a curve (fig. 1) whose equation as found by the method of least squares is

$$\text{Log } P = 1.37 \times 10^{-3}\ Ca + 1.67$$

where P = milligrams of phosphorus retained for a 4-week

[5] The authors are indebted to Dr. Ralph L. Ferguson of the Department of Pathology, University of South Dakota School of Medicine, for microscopic examination of the animals.

TABLE 3

*Ca and P retention of 18 rats on the Steenbock-Black diet* [1]

| WEEK | INTAKE | | EXCRETION | | | | | | RETENTION | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ca | | | P | | | Ca | | P | |
| | Ca | P | Urine | Feces | Total | Urine | Feces | Total | Absolute | % | Absolute | % |
| 1 | 530 | 130 | 120 | 325 | 445 | 2 | 96 | 98 | 85 | 16.0 | 32 | 24.6 |
| 2 | 551 | 136 | 130 | 346 | 476 | 2 | 97 | 99 | 75 | 13.6 | 37 | 27.2 |
| 3 | 565 | 139 | 135 | 368 | 503 | 3 | 106 | 109 | 62 | 11.0 | 30 | 21.6 |
| 4 | 543 | 134 | 128 | 367 | 495 | 4 | 104 | 108 | 48 | 8.8 | 26 | 19.4 |
| Total | 2189 | 539 | 513 | 1406 | 1919 | 11 | 403 | 414 | 270 | 12.3 | 125 | 23.2 |

[1] All values are in terms of mg/rat, except as otherwise noted.

period and Ca = milligrams of calcium retained for a 4-week period.

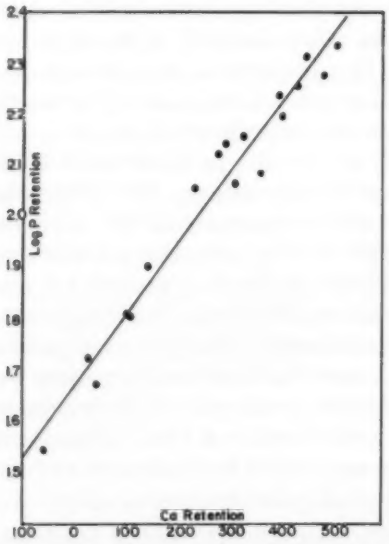


Fig. 1 Ca vs. log P retentions of rats on Steenbock-Black diet. Equation of solid line is log $P = 1.37 \times 10^{-3}$ Ca + 1.67. Values shown are in terms of milligrams retained per rat for a 4-week period.

It might also be noted that in no case from among the 72 weekly phosphorus retention values for the 18 rats on the rachitic diet was a phosphorus loss observed, although in a few cases (9 out of 72) a weekly loss of calcium was noted; three of these 9 involved one rat. It appears, insofar as this study is concerned, that phosphorus can be retained without calcium but that the latter is not retained without the former. It may also be said that for the rats on the Steenbock-Black diet retention was poorest on those diets having the greatest Ca to P ratios.

## SUMMARY AND CONCLUSIONS

The hypothesis originally offered to explain the prevention of rickets in rats on a standard rickets-producing diet when the acid salts of citric and tartaric acids are added, was that an acid-base factor was involved (Hamilton and Schwartz, '33; Shohl, '37). These salts produce an acid medium when in the gastrointestinal tract, thus increasing the solubility of the calcium and hence its absorption. The alkaline ash resulting from the metabolism of these salts provides an environment favorable for bone formation. Shohl ('37), in addition to considering the acid-base factor, suggested the possibility that complex formation might also be involved.

In the present investigation, designed to evaluate the effect of complex formation on calcium retention, the factors of relative acidity and alkalinity were not apparently involved, since the complex-former used and its control material would appear to be neutral in character both in the gastrointestinal tract and upon metabolism in the body. The enhanced calcium retention produced by Am.P. can therefore result insofar as is known only from its complex-forming properties. This bears out the hypothesis of previous investigators that complex formation may be an important factor in calcium metabolism.

Summaries are given of data on the Ca and P retentions of 18 rats maintained on the Steenbock-Black diet, and on the relationship between the Ca and P retentions.

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# THE EFFECT OF MIXED TOCOPHEROLS ON MILK AND BUTTERFAT PRODUCTION OF THE DAIRY COW [1]

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Harris et al. ('47) reported a surprising response of farm milch cows in butterfat and total milk production when daily supplements of 1 gm of mixed tocopherols were fed. An effort has been made to confirm these results and the experimental findings are reported herewith.

## EXPERIMENTAL

The plan of this experiment was designed to make use of a farm herd of pure-bred Holstein-Friesian cattle. Conditions on this farm were average and typical of the management practices of the area. Cattle were handled under the so-called "loose-run" system. The herd was above average in production and had for the previous three years on Official Herd Improvement Registry produced an average in excess of 400 lb. of butterfat per cow. The care, management and feeding of these cattle were directly under the supervision of one of the present authors (P.H.P.). Daily records of milk production were kept, but in all data reported here the HIR monthly

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

The authors are indebted to the Distillation Products Company, Rochester, New York, for the mixed tocopherol preparation generously supplied through the cooperation of Dr. Philip L. Harris.

tests and their estimation of the average daily milk production, butterfat yield and per cent fat were used. This placed the sampling and testing in the hands of disinterested parties, and since these records have official acceptance they seemed to be sufficiently reliable for our use.

The cows were divided into two lots of 9 cows each on the basis of age, stage of lactation, calving record and potential milk production. In most instances daughters of the same bull were divided between lots. Since this herd is a line-bred herd, the variable due to breeding was somewhat reduced. One lot served as the control (Lot I) and the other (Lot II) was fed the basal ration plus 21.3 gm of a mixed tocopherol preparation, which furnished 1 gm of mixed tocopherols per cow per day (the level fed by Harris et al., '47). This was fed once daily by incorporation with the grain mixture in the evening feed. No difficulty was experienced in getting the cows to eat the mixture.

The ration used was composed of good quality corn silage ensiled in the early dough-to-dough stage of maturity; alfalfa-brome grass hay, field-cured and field-chopped at a theoretical length of 1.75 inches; and a 16% grain ration. The hay was estimated to be composed of 40% alfalfa and 60% brome for the first cutting and 75% alfalfa and 25% brome for the second cutting. The hay was cut at the recommended half-bloom stage and since it was "layered" in the barn it was possible to feed a mixture of first and second cuttings in the ratio of 3:1. The hay was fed ad libitum, mixed in approximately this ratio. The grain mixture was composed of the following, in pounds: ground wheat 400, ground oats 400, dried brewers' grain 200, soybean oil meal 100, wheat bran 100, bone meal 20, and trace mineralized salt 20. The tocopherol content of the ration concentrates was determined by the method of Hines and Mattill ('43). The hay averaged 42 mg, the silage 7 mg and the grain mixture 14 mg of tocopherol per pound on an air-dried, uncorrected-moisture basis. The estimation of the daily tocopherol ingestion through the ration was approximately 1 gm per cow per day (0.99 gm), a figure closely

approximating that found occurring naturally in the ration used by Harris et al. ('47).

Two distinct experimental periods were used. Experiment I was set up with the two lots of 9 cows each, and in it we studied the effect of the incorporation of the mixed tocopherol, as indicated above. Experiment II, beginning at the close of Experiment I, was used to test the effect of mixing the tocopherols directly with the whole grains of the grain mixture at this time of grinding. In Experiment II all cows in both lots were fed the grain mixture with the mixed tocopherols incorporated. The experimental period for Experiment I lasted from December 10, 1947, through March 9, 1948, while Experiment II began March 10, 1948, and carried through for 6 weeks. The second experiment was designed to determine whether an antioxidation effect was present when the mixed tocopherols were placed with the grain mixture before grinding. Ground whole grains are known to heat after grinding and it was thought that the tocopherols might prevent undue oxidation.

## RESULTS

When the results were compiled, it was found that three cows had to be eliminated from the records because of premature "drying off" or other abnormal conditions. The data which show the effect of the feeding of mixed tocopherols upon milk and butterfat production are given in table 1.

TABLE 1

*Effect upon milk and butterfat production of feeding 1 gm daily of mixed tocopherols*

| Lot | No. cows | Average daily milk yield by months | | | | Average daily butterfat | | | | Average estimated butterfat yield by months | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Prior (Nov.) 30 days | After | | | Prior (Nov.) 30 days | After | | | Prior (Nov.) 30 days | After | | |
| | | | Dec. | Jan. | Feb. | | Dec. | Jan. | Feb. | | Dec. | Jan. | Feb. |
| | | *lb.* | *lb.* | *lb.* | *lb.* | % | % | % | % | *lb.* | *lb.* | *lb.* | *lb.* |
| Control | 7 | 45.8 | 42.4 | 42.4 | 38.1 | 3.5 | 3.5 | 3.3 | 3.7 | 48.8 | 45.5 | 43.7 | 40.7 |
| Tocopherol-fed | 8 | 45.4 | 41.9 | 39.4 | 33.4 | 3.5 | 3.5 | 3.6 | 3.5 | 47.9 | 43.7 | 44.3 | 34.7 |

It is quite evident that the addition of 1 gm of mixed tocopherols daily to the ration of these cows did not increase milk production or the total butterfat production per month. There was some variation in the per cent of butterfat but this was well within the normal range, and there was no difference between groups. Paired as these cows were, there was a remarkable similarity in the performances of the two lots.

Table 2 shows the milk and butterfat yields for all cows during Experiment II. Again, there was no increase in either milk or butterfat production for this 6-week period and the per cent of fat was remarkably constant.

TABLE 2

*Effect on both lots of cows of mixing tocopherols with grain mixture at the time of grinding*

| LOT | NO. COWS | AVERAGE DAILY MILK YIELD BY MONTHS | | AVERAGE DAILY BUTTERFAT | | AVERAGE ESTIMATED BUTTERFAT YIELD BY MONTHS | |
|---|---|---|---|---|---|---|---|
| | | March | April | March | April | March | April |
| | | lb. | lb. | % | % | lb. | lb. |
| Control | 4 | 38.1 | 34.0 | 3.4 | 3.5 | 37.8 | 35.2 |
| Tocopherol-fed | 7 | 34.6 | 30.8 | 3.4 | 3.5 | 36.6 | 32.5 |

In order to make certain that the stage of lactation or that age did not affect these results, the data were assembled by paired groups, as is shown in table 3. When the cows were paired on the basis of the stage of lactation there was no difference in milk production between the controls and the tocopherol-fed lot. When paired by age, either at the first calf heifer stage or at the mature cow level, the differences again were not significant. The drop in production was very similar in both groups and in most cases the controls produced as well or better than the cows fed the tocopherol preparations. Any differences appearing in the data are due largely to individual variations during the period studied. If the butterfat production and per cent of butterfat are examined on the basis of pairing by stage of lactation or by

age, as in the case of milk production, no significant differences are noted (table 4). At the end of the first experimental period complete blood analyses for vitamin A and carotene

TABLE 3

*Effect of feeding tocopherols on the average daily milk production by months*

| LOT | NO. OF COWS | AVERAGE PRIOR 30 DAYS | EXPERIMENTAL PERIOD (MONTH) | | |
|---|---|---|---|---|---|
| | | | 1 | 2 | 3 |
| | | *lb.* | *lb.* | *lb.* | *lb.* |
| Cows paired by stage of lactation (8–10 weeks) | | | | | |
| Control | 6 | 46.8 | 44.0 | 44.0 | 41.0 |
| Tocopherol-fed | 6 | 45.7 | 44.4 | 42.3 | 37.9 |
| Cows paired by age (2-year olds) | | | | | |
| Control | 3 | 40.5 | 39.7 | 39.6 | 38.1 |
| Tocopherol-fed | 2 | 37.2 | 37.0 | 34.7 | 32.6 |
| Cows paired by age (4–5 years) | | | | | |
| Control | 3 | 53.0 | 48.2 | 48.4 | 43.9 |
| Tocopherol-fed | 4 | 49.9 | 45.5 | 46.0 | 40.5 |

TABLE 4

*Effect of tocopherol feeding on the average monthly butterfat production and average fat content of milk*

| LOT | NO. OF COWS | AVERAGE MONTHLY BUTTERFAT PRODUCTION | | | | AVERAGE FAT CONTENT OF MILK | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Ave. prior 30 days | Experimental period in months: | | | Ave. prior 30 days | Experimental period in months: | | |
| | | | 1 | 2 | 3 | | 1 | 2 | 3 |
| | | *lb.* | *lb.* | *lb.* | *lb.* | | % | % | % |
| Cows paired by stage of lactation (8–10 weeks) | | | | | | | | | |
| Control | 6 | 50.1 | 47.5 | 44.9 | 43.1 | 3.6 | 3.5 | 3.3 | 3.7 |
| Tocopherol-fed | 6 | 49.6 | 44.6 | 47.0 | 39.8 | 3.6 | 3.5 | 3.5 | 3.6 |
| Cows paired by age (2 years old) | | | | | | | | | |
| Control | 3 | 42.3 | 41.5 | 38.8 | 39.0 | 3.5 | 3.5 | 3.2 | 3.6 |
| Tocopherol-fed | 2 | 37.9 | 39.1 | 35.0 | 35.0 | 3.4 | 3.4 | 3.5 | 3.8 |
| Cows paired by age (4 to 5 years old) | | | | | | | | | |
| Control | 3 | 54.8 | 53.6 | 50.6 | 47.3 | 3.8 | 3.6 | 3.4 | 3.7 |
| Tocopherol-fed | 4 | 55.4 | 47.3 | 51.8 | 42.5 | 3.7 | 3.4 | 3.6 | 3.6 |

were made on the entire herd. The control group averaged 25.2 μg %, as compared with the tocopherol-fed group with 21.4 μg %. Both of these groups were well above the marginal level of 15 μg % and were, therefore, adequately supplied with vitamin A. The carotene content was ample in both instances.

A pooled sample of blood, representing 0.2 ml of blood plasma for each cow in the lot, was taken at the close of the first experiment and the tocopherols estimated according to the method of Mayer and Sobotka ('42). The control group averaged 0.70 mg % tocopherol, as compared to the tocopherol-fed lot with 0.68 mg %. Apparently the feeding of mixed tocopherols did not raise the tocopherol content of the blood of these animals.

It is apparent from these data that we were unable to confirm the work of Harris et al. ('47), in that we could not produce an increase in either milk production, butterfat production or the per cent of butterfat with the ration used. It is possible that the character of the ration, particularly the grain portion, differs sufficiently from that used by Harris et al. to account for the difference in results.

## SUMMARY AND CONCLUSIONS

An experiment was set up in which a preparation containing 1 gm of mixed tocopherols was fed to milch cows to determine its effect upon milk and butterfat production. The results indicate definitely that the inclusion in the ration of 1 gm of mixed tocopherols per cow daily does not influence the milk or butterfat yield, and neither does it affect the per cent of butterfat present in the milk. These data do not substantiate the work of Harris et al. ('47), at least when the mixed tocopherols are fed under the conditions prevailing during the present experiment.

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# NITROGEN BALANCE INDEX AND SPECIFIC DYNAMIC ACTION IN RATS RECEIVING AMINO ACID MIXTURES LOW IN ISOLEUCINE, METHIONINE OR VALINE [1,2]

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ONE FIGURE

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The quality of a diet may be measured in terms of efficiency of utilization of energy, nitrogen or some other nutrient. Biological value and nitrogen balance index are measures of efficiency of utilization of nitrogen, and are preferable to body weight observations for the reasons given by Mitchell ('44). The efficiency of utilization of energy of the diet can be expressed as the "net availability of metabolizable energy" (NAME) (Mitchell, '34):

$$\text{NAME} = 1 - \frac{\text{SDA}}{\text{ME}} \qquad (1)$$

SDA is specific dynamic action and ME is metabolizable energy. Since ME is that portion of the total energy intake which is not lost in the excreta, and SDA is the wasted portion

[1] Many of the data of this paper are taken from a thesis submitted by Joseph T. Anderson ('47) to the faculty of the Graduate School of the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] This investigation was supported in part by grants from Swift and Company and from the Navy Department Office of Naval Research. Acknowledgment is made to Merck and Company, Hoffman-LaRoche, Inc., and the Lederle Laboratories for gifts of amino acids and vitamins, amino acids, and folic acid, respectively.

of ME, the above expression gives the fraction available for basal metabolic rate, growth, activity and other productive processes.

In a series of diets fed so as to give equal ME, the one having the highest NAME will produce the lowest SDA. It follows, therefore, that among diets of equal metabolizable energy, specific dynamic action is an inverse measure of efficiency of energy utilization. Mitchell ('34) stated that the best diet for energy retention is the one which yields the highest NAME. Hamilton ('39) and Forbes and Swift ('44) demonstrated that there is an optimum dietary content of vitamins, minerals, protein and fat, and that diets which are not the optimum in these respects are not utilized by the animal at maximum efficiency.

Gross deficiency of any essential amino acid evidently causes a considerable decrease in efficiency of utilization of both energy and nitrogen, since animals may continue to eat such a deficient diet yet lose weight drastically. It may be expected, then, that a diet which is only mildly deficient in one essential amino acid will show a small decrease in efficiency of both nitrogen and energy utilization.

The purpose of our investigation, the first portion of which is described below, was to determine quantitatively by means of N balance index and specific dynamic action the influence of diets, mildly deficient in one essential amino acid, upon the efficiency of utilization of nitrogen and energy for maintenance of the mature rat.

## METHODS

### *Nitrogen metabolism*

Each group of experimental animals consisted of 10 to 12 mature male albino rats. The maximum differences in weight and age were 50 gm and 15 days, respectively. A single group of rats served for three to 5 consecutive experiments. Series 20 rats were 34 weeks old and series 40 rats 24 weeks old at the time of feeding the first amino acid mixture diet. The sequence of feedings in a single cycle were: (1) two or three

weeks of recovery diet; (2) one week of N-free diet; (3) one week of amino acid mixture diet; (4) one and one-half days of fasting. This cycle was repeated as often as seemed desirable. The arrangement of the diets was intended to bring the average body weight of the animals to the same value at the beginning of each amino acid diet period. The N-free period served to deplete the protein stores of the rats to a reproducible extent before each amino acid feeding.

TABLE 1

*Composition of diets*

| COMPONENT | COMPLETE AMINO ACID DIET | N-FREE DIET | RECOVERY DIET |
|---|---|---|---|
| | | *per 100 gm diet* [1] | |
| "Complete" amino acid mixture | 4.19 gm | | |
| Dried whole egg [2] | | | 20.1 gm |
| Sucrose | 65.5 gm | 69.7 gm | 58.3 gm |
| Cottonseed oil (Wesson) | 17.4 gm | 17.4 gm | 8.75 gm |
| $SiO_2$ [3] or $Fe_2O_3$ for marker | 2.3 gm | 2.3 gm | 2.3 gm |
| Salt mixture (Wesson, '32) | 4.5 gm | 4.5 gm | 4.5 gm |
| Cellu flour | 5.8 gm | 5.8 gm | 5.8 gm |
| N from amino acids or egg | 0.53 gm | 0 gm | 1.53 gm |
| Estimated physiological heat value | 437 Cal. | 437 Cal. | 424 Cal. |

[1] One hundred grams of each diet contained 0.92 mg thiamine hydrochloride, 0.92 mg pyridoxine hydrochloride, 1.15 mg methyl naphthoquinone, 1.84 mg riboflavin, 4.6 mg niacin amide, 5.1 mg calcium pantothenate, 4.6 mg alpha tocopherol, 112 mg Navitol containing 65,000 A and 13,000 D units per gram, 115 mg choline chloride and, in the series 40 experiments, also 0.06 mg biotin, 0.06 mg folic acid and 23 mg inositol.

[2] The dried whole egg contained 7.60% N and 40.5% fat.

[3] Isco blanc rouge, Innis Speiden and Co., New York.

The composition of the diets is given in tables 1 and 2. Series 20 rats were fed material furnishing 37.7 Cal./day/rat of estimated physiological heat value (metabolizable energy), the quantity being the same for every rat and in every period. Metabolizable energy was estimated as 4, 9 and 4 Cal. per gram of protein, fat and carbohydrate, respectively. Series 40 rats were larger and were allowed 40.1 Cal./day/rat. The

feeding at the specified caloric intake of the recovery diet, which contained 9.6% protein, permitted a slow gain in weight.

The N intake from the amino acid diets was a little less than the N excretion of the rats on the N-free diet. This resulted in a negative N balance in all amino acid diet periods which, according to the observations of Allison and Anderson ('45) with dogs, insured a maximum N balance index.

TABLE 2

*Amino acid composition of egg and of diets*

| AMINO ACID | WHOLE EGG | | "COMPLETE" AMINO ACID MIXTURE | | |
|---|---|---|---|---|---|
| | Dried whole egg [1] | Acetone-ether insoluble [2] | Natural isomer present | Isomer used | Total amino-acid used |
| | *mg amino acid N/gm total N* | | | | |
| Arginine | 128 | 195 | 192.3 | L | 192.3 |
| Histidine | 36 | 62 | 49.4 | L | 49.4 |
| Isoleucine | 53 | 47 | 45.7 | DL | 91.4 |
| Leucine | 61 | 65 | 63.4 | L | 63.4 |
| Lysine | 86 | 94 | 81.8 | L | 81.8 |
| Methionine | 24 | 23 | 22.7 | DL | 45.4 |
| Phenylalanine | 33 | 32 | 32.0 | DL | 63.9 |
| Threonine | 36 | 36 | 35.8 | DL | 71.7 |
| Tryptophane | 13 | 14 | 14.2 | DL | 28.3 |
| Valine | 55 | 54 | 53.9 | DL | 107.8 |
| Glutamic acid | | | 204.5 | L | 204.5 |
| Total N | 1000 | 1000 | | | 1000 |

[1] Mitchell and Block ('46).
[2] Edwards et al. ('46).

Table 2 gives the composition of the "complete" amino acid mixture for comparison of its essential amino acids with two analyses of whole egg protein. The amount of each natural form of amino acid included in the "complete" amino acid mixture, except for some discrepancy in the cases of histidine and lysine, is close to that in the analysis reported by Edwards et al. ('46). Glutamic acid was added to make the total N the same as in egg. This mixture, therefore, resembled egg protein very closely in respect to its content of the natural isomers of the essential amino acids. The non-essential amino acids

in egg were replaced in the mixture by the unnatural forms plus glutamic acid.

Another amino acid mixture called the "low-isoleucine" mixture differed from that shown in table 2 in that the amount of DL-isoleucine was reduced to one-third. The total N content was kept constant by the addition of more glutamic acid. In analogous fashion "low-methionine" and "low-valine" mixtures were prepared. A "complete" amino acid mixture was used also in which the glutamic acid was replaced by glycine containing the same amount of N. Since the physiological heat values of all the amino acid mixtures were close to that of sucrose, any change in the total weight of amino acids used per 100 gm of diet was compensated for by an equal and opposite change in weight of sucrose.

Since some rats failed voluntarily to eat the prescribed quantity of N-free diet for 7 days, this diet, as well as the amino acid diets, was fed every 12 hours by stomach tube. The dry diet was mixed thoroughly with about 35% of water and injected from a pump through a no. 8 French-size Wishart catheter. The brass pump used was a gasoline blowtorch air pump in which the piston was sealed by a leather washer. Stops placed on the piston shaft limited its travel to give the desired volume. The pump, the catheter and a storage reservoir were connected to a three-way metal stopcock in such a way that the syrupy diet could be forced by air pressure into the pump and subsequently discharged from the pump through the catheter.

Nitrogen intake was determined by analysis of portions of diet exactly like those given the rats and delivered from the same catheter. The feces for each diet were separated by giving $Fe_2O_3$-marked diet at the first feeding of each dietary period, and by giving a mixture of $Fe_2O_3$, agar and cellu flour by stomach tube on the first morning of the fast. Urine was collected at the end of the third, 5th and 7th days of the N-free and amino acid diet periods by washing out the cage with hot water a few minutes after feeding the rat. Nitrogen

determinations were carried out by the micro-Kjeldahl method of Miller and Houghton ('45).

*Energy metabolism*

The heat production of the rats was measured during the greatest possible part of the daylight half of the last day on each amino acid diet and again for a similar time period 48 hours later, after a 36-hour fast. Each rat was placed in a body size screen cage, so small that the rat could not turn around. Each cage rested in a stainless steel box which collected the urine passed during the test. Six cages were placed together in a battery jar, which was sealed and made part of a Haldane small animal metabolism train. Two $CO_2$ absorbing units were provided. One of these was used only when the rats were quiet, and the other for the remainder of the time.

After equilibration with a stream of dry $CO_2$-free air at 10 l/min., the battery jar containing the rats was weighed at about 10:30 A.M. and then connected so that all the $H_2O$ and $CO_2$ given off by the rats was collected until about 8:30 P.M. The rate of air flow was 10 l/min. at all times. The rats were observed for extended periods; when they were all quiet the air stream was changed to the "quiet" $CO_2$ absorber, but it was returned to the "active" $CO_2$ absorber as soon as any one of them had been active for one and one-half to two minutes. Approximately one hour of quiet period was accumulated during each third of the 10-hour total period. The average R.Q. for the entire period was computed from the total $CO_2$ production and $O_2$ consumption. This R.Q. together with the quiet period $CO_2$ production and the urinary N excretion rate was used to compute the resting heat production rate. A similar method was used by Forbes and Swift ('44).

Since our purpose was to determine the influence of the amino acid composition of the diet on the heat production, all the other factors known to change heat production were controlled.

The environmental temperature during the test was held between 28 and 31°C. and between 26 and 29°C. during the remainder of the experiment. Activity cycles which are known to cause metabolic rate variations were regulated by: (1) using males, which are free from estrous cycles; (2) regularly keeping the room light from 9 A.M. to 9 P.M. and dark from 9 P.M. to 9 A.M.; and (3) feeding the animals daily at 9 A.M. and 9 P.M. The body size was kept substantially constant. The caloric intake was constant from day to day except during the fast. The size of the meal administered at the beginning of the test was always the same. Although activity during the heat production test was controlled as described above, this factor was probably the most important source of variability in the heat production values.

## RESULTS

### *Nitrogen metabolism*

Three experiments were carried out with the rats of series 20, testing, in the order named (1) the "complete" amino acid mixture, (2) the low isoleucine mixture, and (3) the "complete" amino acid mixture. The average N balance data are given in table 3. The urine N values are from the last 4 days of each 7-day diet period. These gave a smaller standard deviation of N balance index than urinary N from the last two days only, and there was no consistent difference between the average urinary N of the 6th and 7th days and the average urinary N of the 4th and 5th days.

Nitrogen balance index was defined by Allison and Anderson ('45). All experimentally-determined biological values in which endogenous nitrogen excretion is estimated on the basis of total nitrogen excretion on a nitrogen-free diet are really nitrogen balance indices. We accept the suggestion of Allison, Seeley, Brown and Anderson ('46) that the term "biological value" be reserved for the fraction of absorbed nitrogen which is retained according to computations based

TABLE 3

*Average nitrogen balance data for rats receiving amino acid mixture diets*

| | SERIES 20 | | | SERIES 40 | | | | |
|---|---|---|---|---|---|---|---|---|
| EXPERIMENT NUMBER | I | II | III | I | II | III | IV | V |
| NUMBER OF RATS | 10 | 9 | 8 | 12 | 12 | 12 | 11 | 10 |
| BODY WEIGHT [1] (KG) | 0.212 | 0.212 | 0.221 | 0.230 | 0.232 | 0.231 | 0.231 | 0.234 |
| METABOLIC BODY SIZE ($KG^{3/4}$) | 0.312 | 0.312 | 0.322 | 0.332 | 0.334 | 0.333 | 0.333 | 0.336 |
| BODY SURFACE [2] ($M^2$) | 0.0314 | 0.0314 | 0.0321 | 0.0328 | 0.0330 | 0.0328 | 0.0328 | 0.0331 |
| | N balance data (mg N/day/$kg^{3/4}$) | | | | | | | |
| "N-free" period | | | | | | | | |
| NI' (Nitrogen intake) | 5 | 4 | 5 | 10 | 6 | 6 | 6 | 6 |
| FN' (Feces nitrogen) | 45 | 42 | 38 | 40 | 39 | 38 | 41 | 36 |
| UN' (Urine nitrogen) | 194 | 202 | 164 | 144 | 140 | 139 | 155 | 145 |
| Amino acid period | | | | | | | | |
| Amino acid mixture | Complete | Low-iso-leucine | Complete | Complete | Comp. + glycine | Low meth-ionine | Low valine | Complete |
| NI (Nitrogen intake) | 152 | 151 | 145 | 156 | 146 | 141 | 142 | 139 |
| FN (Feces nitrogen) | 41 | 39 | 39 | 37 | 34 | 34 | 37 | 33 |
| UN (Urine nitrogen) | 127 | 177 | 122 | 131 | 117 | 202 | 128 | 125 |
| $NE_o$ (N excretion at zero NI) | 242 | 246 | 203 | 185 | 180 | 175 | 196 | 182 |
| Stand. dev. of mean $NE_o$ | 6 | 8 | 8 | 4 | 10 | 5 | 9 | 3 |
| $AN_o$ (Absorbed N to give equilibrium) | 168 | 212 | 156 | 171 | 155 | 323 | 163 | 159 |
| Stand. dev. of mean $AN_o$ | 4 | 6 | 3 | 4 | 3 | 15 | 4 | 4 |
| $P_1$ for $AN_o$ [3] | | $< 0.0001$ | 0.02 | | 0.004 | $< 0.0001$ | 0.18 | 0.04 |
| $P_2$ for $AN_o$ [3] | | $< 0.0001$ | | | 0.4 | $< 0.0001$ | 0.5 | |
| D (True digestibility) | 1.03 | 1.02 | 0.99 | 1.02 | 1.03 | 1.03 | 1.03 | 1.02 |
| K (N balance index of absorbed N) | 1.44 | 1.17 | 1.30 | 1.09 | 1.16 | 0.55 | 1.18 | 1.15 |
| Stand. dev. of mean K | 0.04 | 0.05 | 0.04 | 0.02 | 0.06 | 0.03 | 0.04 | 0.02 |
| $P_1$ for K [3] | | 0.001 | 0.04 | | 0.30 | $< 0.0001$ | 0.09 | 0.09 |
| $P_2$ for K [3] | | 0.08 | | | 0.9 | $< 0.0001$ | 0.5 | |

[1] Average body weight at end of amino acid diet period.

[2] Formula (2) of Lee ('29): $(M^2) = 0.001254\ (gm)^{0.6}$.

[3] Probability that the differences between mean values might have arisen due to chance only, computed from the Student "t" value (Kenney, '39). $P_1$ is for comparison of the period indicated with the *preceding* "complete" amino acid period. $P_2$ is for comparison of the period indicated with the *following* "complete" amino acid period.

on the true endogenous N excretion. Biological value can be obtained from the expression

$$NB = (BV)\ (AN) - EN \qquad (2)$$

in which EN is the true endogenous N excretion, AN is absorbed N, NB is N balance and BV is biological value. The nitrogen balance index is K in the following equation:

$$NB = K\ (AN) - NE_o \qquad (3)$$

in which $NE_o$ is N excretion on a N-free diet. Since true endogenous N probably cannot be determined, neither can biological value as defined by Allison et al. The function obtained experimentally is N balance index.

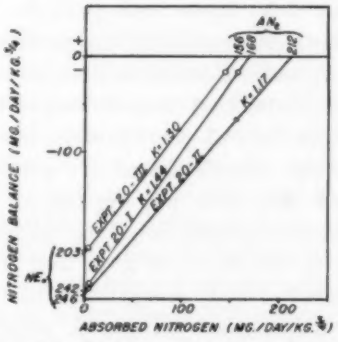


Fig. 1 N balance experiments with series 20 rats fed amino acid mixtures. The low isoleucine diet of experiment 20-II gives a low N balance index (slope of the line = K) and a high requirement of absorbed N for equilibrium ($AN_\infty$) compared with the complete diet of experiments 20-I and 20-III.

In figure 1 the results of the three experiments of series 20 are presented as plots of N balance against absorbed N. Each experiment yields two points, the lower one from the N-free diet and the upper one from the amino acid diet. Since Allison and Anderson ('45) have shown that all such points fall on a straight line conforming to the relationship expressed in equation (3), lines have been drawn through each pair of points. The slope of each line is the N balance index of ab-

sorbed N, which is K in equation (3). The lower intercept with opposite sign is $NE_o$, the N excretion at zero N intake. The upper intercept, $AN_e$, is the absorbed N necessary to give N equilibrium or the maintenance N requirement for the amino acid mixture used. The relationship among these quantities is obtained by making NB equal to 0 in equation (3).

$$K = \frac{NE_o}{AN_e} \qquad (4)$$

The $AN_e$ value for a diet is determined chiefly by the N balance data of the amino acid diet period and is only slightly influenced by the N-free period, as can be appreciated from the lines plotted in figure 1. K, on the other hand, is dependent equally on the data from both periods. Thus, although $AN_e$ and K are both measures of the quality of the diet for promotion of N retention and are both computed from the same experimental data, they may be expected to give somewhat different indications of quality, since the relative weights of the data in the two computations are quite different.

N balance index (K) does not depend on body size. $AN_e$ and $NE_o$ are directly proportional to metabolic body size, which, according to Kleiber ('47), is well expressed by the three-quarters power of body weight. Unless some qualitative difference in N metabolism exists, N requirements for N equilibrium expressed as mg N/day/$kg^{3/4}$ should be nearly the same for large and small rats, as well as for various species.

The N balance index for the low isoleucine diet was 1.17, as compared with 1.44 and 1.30 for the "complete" amino acid mixture diet. The difference is highly significant in one case and of doubtful significance in the other, as indicated by probabilities of 0.001 and 0.08. The $AN_e$ for the low isoleucine diet was 212 mg N/day/$kg^{3/4}$, as compared with 168 and 156 for the "complete" amino acid mixture diet. These differences are both highly significant. It is therefore concluded that the low isoleucine amino acid mixture is utilized by the

rat for maintenance of N equilibrium less efficiently than the "complete" amino acid mixture.

An abnormal appearance of the eyes of the rats began to appear in experiment 20-II and was quite evident in experiment 20-III, at which time it was diagnosed as "spectacle-eye" due to biotin deficiency. At the end of experiment 20-III the rats were placed on the recovery diet supplemented with 5 μg of biotin per rat per day, and at the end of three weeks the eyes had definitely improved and hair had begun to grow in the previously bald areas. Apparently a diet containing dried whole egg needs supplementation with biotin. Although it is possible that the biotin deficiency may have influenced the N balance and energy metabolism, there is no indication of it in the data.

The series 40 rats, which unfortunately came from a different colony, were used to compare the "complete" amino acid mixture with three other mixtures. The only difference in the dietary amino acid mixture of period 40-II was the isonitrogenous substitution of glycine for glutamic acid. The "complete with glycine" mixture gave a N balance index (K) which was not significantly different from the one obtained in either the preceding or following trials with the "complete" amino acid mixture containing glutamic acid. Comparison of $AN_e$ values indicates a significant difference between 40-I and 40-II but not between 40-II and 40-V. In spite of one discordant result in 4, it is concluded that substituting glycine for glutamic acid in amounts containing equal N causes no change in efficiency of utilization of N of the amino acid mixture.

A low methionine amino acid mixture in which 1.51% of the N was provided by DL-methionine was fed in experiment 40-III. The N balance index was 0.55, which is a most striking decrease from the values of 1.09 and 1.15 for the "complete" amino acid mixture. The amount of absorbed N necessary to give equilibrium when feeding the low methionine amino acid mixture was found by extrapolation to be 323 mg N/day/$kg^{3/4}$, which is markedly higher than the 171 and

159 mg computed for the "complete" amino acid mixture. The low N balance index and high $AN_e$ both indicate that the amount of methionine in the low methionine diet is so small as to depress the efficiency of utilization of N of the amino acid mixture.

In experiment 40-IV the valine was reduced to one-third of the amount in the "complete" amino acid mixture, so that 3.59% of the N was supplied by DL-valine. There was no significant difference between the N balance index and $AN_e$ in this period and the values in the preceding or following "complete" amino acid periods. It is concluded that the low valine amino acid mixture provides enough valine to support full efficiency of N utilization.

### *Energy metabolism*

In connection with each amino acid diet the resting postcibal metabolic rate and the resting fasting metabolic rate of the rats were determined as described above. The metabolic rate values are listed in table 4 and are to be understood as values obtained during about three hours of observed rest distributed fairly uniformly through the light half of a day and computed on a 24-hour basis. The two values which are subtracted to give the specific dynamic action were determined on the same individuals, with a time interval of only 48 hours between the two tests. The results are expressed as Cal./day/rat, since each animal in a given series received the same amount of diet regardless of individual or time variations in body weight. The SDA values in each series of experiments represent, therefore, the average effect of equal amounts of estimated metabolizable energy in rats of varying body weights.

As is indicated in table 4, the SDA of the low isoleucine diet, 6.0 Cal./day/rat, was found to be significantly greater than the 2.6 and 3.1 Cal./day/rat for the "complete" amino acid diet. This indicates that decreasing to one-third the isoleucine in the "complete" amino acid mixture caused a decrease in efficiency of utilization of metabolizable energy, as well as

TABLE 4

*Average resting energy metabolism data for rats receiving amino acid mixture diets*

| EXPERIMENT NUMBER | SERIES 20 | | | SERIES 40 | | | | |
|---|---|---|---|---|---|---|---|---|
| | I | II | III | I | II | III | IV | V |
| Diet | Complete | Low-iso-leucine | Complete | Complete | Comp. + glycine | Low methionine | Low valine | Complete |
| Estimated metabolizable energy (Cal./day/rat) | 37.7 | 37.7 | 37.7 | 40.1 | 40.1 | 40.1 | 40.1 | 40.1 |
| Number of rats | 10 | 10 | 8 | 12 | 12 | 12 | 11 | 11 |
| Body wt.[1] (kg) | 0.212 | 0.212 | 0.221 | 0.230 | 0.232 | 0.231 | 0.231 | 0.234 |
| Respiratory quotients: | | | | | | | | |
| Receiving diet | 0.96 | 0.93 | 0.95 | 0.97 | 0.96 | 0.95 | 0.97 | 0.94 |
| After 36-hr. fast | 0.71 | 0.73 | 0.73 | 0.73 | 0.71 | 0.73 | 0.72 | 0.72 |
| Metabolic rates (Cal./day/rat): | | | | | | | | |
| Receiving diet | 23.1 | 27.7 | 24.7 | 27.2 | 25.8 | 26.6 | 27.6 | 26.4 |
| After 36-hr. fast | 20.5 | 21.7 | 21.6 | 23.7 | 24.4 | 24.4 | 23.4 | 24.1 |
| SDA | 2.6 | 6.0 | 3.1 | 3.5 | 1.4 | 2.2 | 4.2 | 2.3 |
| Standard dev.[2] of mean SDA | 0.8 | 0.8 | 0.8 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| $P_1$[3] | | 0.006 | 0.7 | | 0.15 | 0.4 | 0.6 | 0.4 |
| $P_2$[3] | | 0.02 | | | 0.6 | 1.0 | 0.2 | |

[1] Mean body weight at end of amino acid diet period.

[2] A single estimate of the standard deviation of a mean SDA was computed for each series from all the differences found on various days between the average metabolic rates of the two matched groups in the two metabolism units.

[3] Probabilities that the differences between mean SDA values occurred by chance. $P_1$ is for comparison with the preceding "complete" amino acid period and $P_2$ is for comparison with the following "complete" amino acid period.

a decrease in efficiency of utilization of absorbed N. No significant alteration in SDA appeared with other changes in the amino acid composition of the diet, although by analogy with the N balance data the low methionine diet should have caused an increase in SDA.

## DISCUSSION

### *Body N-sparing effect*

The data shown in table 3 indicate that in every experiment except that involving the low methionine diet the urinary N excretion was decreased when an amino acid mixture was introduced into a N-free diet. This fact accounts for a N balance index greater than unity. As pointed out by Allison, Seeley, Brown and Anderson ('46), the N balance index must be higher than the biological value and the N excretion at zero N intake ($NE_o$) must be greater than the true endogenous N excretion (EN) in such a circumstance.

Nielson, Gerber and Corley ('39) found that the incorporation of L-cystine into the N-free diet of dogs caused the urinary N to be decreased on the day of the treatment and for a few days thereafter. They also observed a similar response after administering a mixture containing all the essential amino acids except threonine. Miller ('44) observed that the addition of methionine or cystine to a very low protein diet caused a 30 to 45% reduction in N excretion. Allison, Seeley, Brown and Anderson ('46) and Allison, Anderson and Seeley ('47) demonstrated that feeding methionine, methionine-supplemented proteins and unsupplemented egg white protein diminished the urinary N in the dog and that the effect was more marked the longer the preliminary period of protein-free feeding. Urinary N in the rat is decreased by supplementing a N-free diet with any of the following: methionine, cystine, arginine, isoleucine, histidine, choline or a mixture of the 10 essential amino acids (Brush, Willman and Swanson, '47). According to Johnson, Deuel, Morehouse and Mehl ('47) and Cox et al. ('47), methionine feeding does not reduce urinary N in man even after protein depletion.

### *Minimum urinary N*

Evidently the urinary N excretion of the rat on a N-free diet is altered considerably by variations in protein depletion or by the addition to the diet of small amounts of certain amino acids. In the data shown in table 3 the $NE_o$ values for a single diet are observed to differ among experiments much more than do the $AN_e$ values. Since $K = NE_o/AN_e$, variability in $NE_o$ is transmitted directly to K. In comparing experiments 20-I, 20-III, 40-I, 40-II, 40-IV and 40-V, $AN_e$ is seen to be a much more consistent attribute of the diet than K. It is suggested that the use of urinary N excretion on a N-free diet in computing N balance index in rats leads to difficulties in two ways: (1) N balance indices are higher than true biological values and (2) variability in N balance index occurs among groups of animals and between dietary periods with the same group. An alternative procedure is to feed the test protein or amino acid mixture at two levels.

The mean urinary N excretion in 6 experiments with the N-free diet was $157 \pm 8$ mg/day/kg$^{3/4}$. The addition of an adequate amino acid mixture to this basic, N-free diet resulted in a highly significant diminution in urinary N excretion. In the same 6 experiments in which the "complete" amino acid mixture or its equivalent was fed, the urinary N excretion was reduced to $125 \pm 2$ mg/day/kg$^{3/4}$. It seems likely that refinements in the amino acid mixture may reduce this further so that the present value should therefore not be considered the absolute minimum. If related to the energy metabolism, it represents an excretion of 1.79 mg N per basal Calorie, which is lower than previously published values for the rat (Smuts, '35: 1.988 mg; Bricker and Mitchell, '47: 2.16 mg) and somewhat higher than recently reported values for man (Hawley, Murlin, Nasset and Szymanski, '48: 1.19 mg for men and 1.32 mg for women). It is interesting also that in 7 of the 8 experiments (table 3), feeding amino acid mixtures reduced the fecal N as compared with the corresponding N-free periods.

## SUMMARY AND CONCLUSIONS

Simultaneous nitrogen balance and energy metabolism studies were conducted on adult rats receiving amino acid mixtures in a diet otherwise very low in nitrogen. Each amino acid diet period was preceded by a 7-day depletion period in which an isocaloric N-free diet was fed. The diets were administered by stomach tube twice daily in order to insure equal intake by every animal every day. Resting energy metabolism determinations were made during a 10-hour period on the last day of the amino acid diet and again two days later after a fast of 36 hours. The difference between these rates was taken as representing the specific dynamic action of the diet.

When a "complete" amino acid mixture simulating whole egg protein was fed the N balance index was invariably greater than unity. The N requirement for maintenance on this amino acid mixture averaged 162 mg N/day/kg$^{3/4}$. In 6 experiments with two groups of rats the maintenance N requirement proved a more consistent characteristic of the amino acid mixture than the N balance index.

Reducing the DL-isoleucine to one-third the quantity in the "complete" amino acid mixture caused a decrease in N balance index, an increase in maintenance N requirement to 212 mg N/day/kg$^{3/4}$, and an increase in specific dynamic action of 3 Cal./day/rat. A similar reduction in DL-methionine caused a decrease of N balance index to about half and an increase of maintenance N requirement to 323 mg N/day/kg$^{3/4}$ but no significant change in specific dynamic action. Reduction of DL-valine to one-third the quantity in the "complete" amino acid mixture caused no significant change in either N utilization or energy metabolism. Substituting glycine for glutamic acid caused no significant change in N balance index, maintenance N requirement or specific dynamic action.

Reducing the quantity of an essential amino acid will, if carried far enough, lead to decreased efficiency of utilization of N (decreased N balance index and increased maintenance N requirement), and probably to a parallel decrease in effi-

ciency of energy utilization (increased specific dynamic action). Such a decrease in efficiency of N utilization was found when either isoleucine or methionine was reduced to one-third. The corresponding decrease in efficiency of energy utilization was found only in the case of the low isoleucine diet.

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# THE PLACENTAL AND MAMMARY TRANSFER OF TOCOPHEROLS (VITAMIN E) IN SHEEP, GOATS AND SWINE

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Willman et al. ('45, '46) have shown that when ewes are fed a ration consisting of legume hay and red kidney beans during gestation and lactation, a high percentage of the suckling lambs develop muscle dystrophy ("stiff-lamb disease"). The pathological picture appears to be identical with the vitamin E deficiency seen in laboratory animals (Willman et al., '34; Mackenzie et al., '41) and the disease can be prevented or cured in the early stages by feeding alpha-tocopherol. Mason and Bryan ('38, '40) found that vitamin E supplementation increased the stores of vitamin E in the young and the milk of rats. Other investigators (Evans and Burr, '38; Olcott, '38; Pappenheimer, '42) have shown that the young of vitamin E-deficient rats and mice developed symptoms of vitamin E deficiency during the first few weeks of life, while young from normally-fed females showed no such symptoms. More recently, Parrish et al. ('47) have shown that supplementing the ration of dairy cows with 500 to 1000 mg of tocopherols daily during the last 4 weeks of pregnancy increased the tocopherol content of the colostrum from 107 to 150 μg per gram of fat, and that feeding 10 gm of tocopherols daily increased the colostral content to 487 μg per gram of fat.

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These studies suggested that the incidence and severity of the dystrophy mentioned above might be influenced by the placental and mammary transfer of alpha-tocopherol. Because of the lack of published data on this subject, it seemed worthwhile to make a study using farm animals.

## EXPERIMENTAL PROCEDURE

Forty ewes, 16 goats and 25 sows were used in this study. The ewes and goats were fed a ration consisting of second-cutting mixed alfalfa and clover hay and red kidney beans. Half of them received, in addition, a daily supplement of 80 mg of mixed tocopherols [2] per 100 lb. of body weight. The pigs were fed a ration of 760 parts of ground yellow corn, 100 of soybean oil meal, 100 of ground alfalfa hay, 30 of meat scrap, and 10 of minerals. Half of the sows received the daily tocopherol supplement of 80 mg per 100 lb. of body weight.

At the time of parturition the tocopherol supplement was removed from the diets. Samples of liver and blood were obtained from the newborn animals within a few hours after birth. Care was taken to use only the young which were removed from their dams before they had sucked. Colostrum samples were taken before the mothers had been nursed. Milk was collected from the same females again 4 days after parturition. The method of Quaife and Harris ('44) that employs the microhydrogenation technique of Quaife and Biehler ('45), was used to determine the tocopherol content of blood plasma. The method of Quaife ('47) was used to determine the tocopherol content of colostrum and milk. The method of Hines and Mattill ('43), modified as indicated below, was used to determine the tocopherol content of livers. Instead of using the florisil XS earth adsorption column for removing interfering compounds after the $H_2SO_4$ and KOH treatments, the sample was taken up in a 50:50 mixture of cyclohexane and alcohol, and hydrogenated. Tocopherols were then measured in the same manner as for plasma and milk except that the

[2] The vitamin E supplement, "Myvadry," was generously supplied by Distillation Products, Inc., Rochester, New York.

absorption reading was taken after a one-minute reaction time. Essentially stable readings were obtained between one and 10 minutes if all light was excluded, indicating, according to Baxter ('47), that probably the non-tocopherol reducing substances had been removed.

## RESULTS AND DISCUSSION

The tocopherol contents of the livers and blood plasma of newborn lambs, kids and pigs are shown in table 1.

While prepartum tocopherol supplementation at the levels used increased the average tocopherol content of the livers of the newborn animals, the increases were not statistically sig-

TABLE 1

*The influence of diet on the tocopherol content of liver and plasma of newborn animals*

| SPECIES | TOCOPHEROL CONTENT | | | |
|---|---|---|---|---|
| | Liver | | Plasma | |
| | Controls | Supplemented | Controls | Supplemented |
| | *μg/gm* | | *μg/100 ml* | |
| Lambs | (6)[1] 25.3 ± 2.7 | (6) 30.0 ± 4.3 | (6) 20 ± 10 | (6) 94 ± 62 |
| Kids | (5) 10.4 ± 1.5 | (6) 13.1 ± 2.0 | (5) 16 ± 8 | (5) 65 ± 40 |
| Pigs | (8) 24.7 ± 3.6 | (10) 26.4 ± 4.7 | (6) 120 ± 28 | (7) 101 ± 33 |

[1] Figures in parenthesis are the numbers of samples analyzed.

nificant (5% level of probability). This may indicate either that liver storage of tocopherol in the newborn cannot be significantly increased by prepartum tocopherol supplementation or that greater amounts are required to cause significant increases. It is also possible that the liver may not be the site of richest tocopherol storage, since Mason ('42) and Abderhalden ('45a) have shown that vitamin E is widely distributed throughout the animal body. It is interesting that Abderhalden reported the liver of the newborn infant to contain 4.60 μg of tocopherols per gram of tissue; two livers of newborn calves analyzed by the authors showed an average tocopherol content of 13.8 μg per gram of fresh liver tissue. No other values

for the tocopherol content of the livers of newborn mammals were found in the literature.

The tocopherol content of the blood plasma of newborn lambs and kids was significantly increased (1% level of probability) by prepartum vitamin E supplementation, but no increase occurred in the pigs (table 1). This may be explained, in part, on the basis that the ration fed to the ewes and goats was deficient in tocopherol activity (Willman et al., '45, '46), whereas the pig's ration was adequate; thus, the former animals might be expected to show a greater response to supplemental tocopherols than the sows. Abderhalden ('45b) reported that the blood plasma of the newborn infant contained

TABLE 2

*The influence of diet on the tocopherol content of colostrum and milk of sheep, goats and pigs*

| SPECIES | TOCOPHEROL CONTENT | | | |
|---|---|---|---|---|
| | Colostrum | | Milk | |
| | Controls | Supplemented | Controls | Supplemented |
| | | *μg/gm fat* | | |
| Sheep | (19)[1] 47 ± 20 | (14) 78 ± 25 | (19) 15 ± 6 | (15) 30 ± 7 |
| Goats | (5) 59 ± 18 | (8) 154 ± 10 | (5) 14 ± 5 | (8) 31 ± 13 |
| Pigs | (10) 186 ± 61 | (10) 399 ± 175 | | |

[1] Figures in parentheses are the numbers of samples analyzed.

an average of 96 μg of tocopherols per 100 ml of plasma. Straumfjord and Quaife ('46) on the other hand reported a plasma content of 340 μg of tocopherols per 100 ml for the newborn infant.

The tocopherol content of the colostrum and milk of the ewe, goat and sow is shown in table 2.

Prepartum tocopherol supplementation significantly increased the tocopherol content of the colostrum of all three species. In the case of the sows and goats, the increase was highly significant (1% level of probability), but the varia-

bility among animals on the same ration was very large. Parrish et al. ('47) made a similar observation from their data on the tocopherol content of cow's colostrum and milk. Sow's colostrum contained about three times as much tocopherol as that of the goats and ewes, and somewhat more than that reported by Parrish et al. ('47) for cows fed a normal ration. Colostrum from ewes fed a more standard ration of mixed hay, corn silage and cereal grains contained an average of 106 μg of tocopherols per gram of fat. Quaife ('47) reported that human colostrum collected during the first week after parturition contained 0.13 to 3.6 mg of tocopherol per 100 ml. Abderhalden ('44) found that human colostrum taken two days after childbirth contained an average of 3.3 mg per 100 gm, and that at 4 to 10 days postpartum the milk contained 0.94 mg per 100 gm.

Milk of the ewe and goat contained approximately one-fourth as much tocopherol as the colostrum of these animals. This is in agreement with the findings of Parrish et al. ('47) for dairy cows. The milk of the ewes and goats that received tocopherol supplements prepartum contained approximately twice as much tocopherol as that of non-supplemented ewes and goats.

## SUMMARY

The placental transfer and colostral storage of total tocopherols was investigated in sheep, goats and swine. Supplementing the prepartum ration with 80 mg of mixed tocopherols per 100 lb. body weight slightly increased the liver storage of tocopherols in the newborn animal, but the increase was not statistically significant. A highly significant increase in the tocopherol content of the blood plasma of the lambs and kids resulted from the prepartum supplementation, but no increase was observed in swine. Prepartum supplementation caused a two-fold increase in the tocopherol content of the colstrum in all species. Colostrum contained three to 4 times as much tocopherol as the milk obtained from the same animals 4 days later.

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# INFLUENCE OF THE CONCENTRATION OF MIXTURES OF VARIOUS COMPONENTS OF THE VITAMIN B COMPLEX ON BIOLOGICAL VALUE OF CASEIN AND ON ECONOMY OF FOOD UTILIZATION [1]

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There is ample evidence in the literature that the various components of the vitamin B complex play significant roles in nitrogen metabolism. Urinary studies of animals subsisting on diets lacking members of the complex show changes in the excretion of ammonia, urea, creatine, uric acid and allantoin, and examination of the blood reveals alterations in concentrations of non-protein nitrogen and other factors (Sure and Dichek, '41; Sure and Ford, '42). The finding by Gunsalus and Bellamy ('44) and Bellamy et al. ('45) that pyridoxine functions as a co-factor in amino acid decarboxylases suggests a role for this B vitamin in protein metabolism. The relationship between tryptophane and niacin studied by Krehl and colleagues ('46a, '46b), Schweigert et al. ('47, '48) and Salmon ('47) can also be cited as pertinent in this connection.

The purpose of the present study was to investigate the influence of the concentration of synthetic mixtures of the

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vitamin B complex on protein efficiency and on economy of total food utilization. The biological values of purified casein [2] fed at different planes of intake and supplemented with graduated increasing concentrations of mixtures of various components of the vitamin B complex were determined by the nitrogen balance methods of Mitchell ('24, '44).

## EXPERIMENTAL

The percentage composition of the rations was as follows: Casein, 8.2 to 20; cellu flour, 2; Sure's salts no. 1 (Sure, '41), 4; lard, 10; cod liver oil, 1.5; wheat germ oil, 0.5; and the balance, cerelose. Synthetic mixtures of pure crystalline components of the vitamin B complex which have proved to be essential for growth of the rat were used. The composition of these mixtures is given in table 1. Six rats were used in each group of experiments. However, the findings on animals which showed the greatest variations from the mean were eliminated, so that in table 2 results are shown on 5 animals and in table 3 on 4 animals. In table 4 results are submitted on 6 animals.

TABLE 1

*Composition of vitamin B complex mixtures*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| Thiamine | 3 μg | 5 μg | 10 μg | 15 μg | 25 μg | 50 μg | 100 μg |
| Riboflavin | 3 μg | 5 μg | 10 μg | 15 μg | 25 μg | 50 μg | 100 μg |
| Pyridoxine | 3 μg | 5 μg | 10 μg | 15 μg | 25 μg | 50 μg | 100 μg |
| Niacin | 3 μg | 5 μg | 10 μg | 15 μg | 25 μg | 50 μg | 100 μg |
| Calcium pantothenate | 25 μg | 50 μg | 75 μg | 100 μg | 150 μg | 300 μg | 600 μg |
| *p*-aminobenzoic acid | 250 μg | 500 μg | 1 mg | 2 mg | 3 mg | 6 mg | 12 mg |
| Inositol | 75 μg | 150 μg | 300 μg | 600 μg | 1 mg | 3 mg | 3 mg |
| Choline chloride | 3 mg | 6 mg | 6 mg | 6 mg | 6 mg | 9 mg | 12 mg |

[2] Smaco.

Urinary and fecal balances were determined over periods of 7 days on an egg standardizing ration and then for 7 days on the experimental rations containing 8.2, 10, and 20% casein; these rations contained 7.1, 8.7, and 17.3% protein, respectively. The 8.2 and 10% casein rations were supplemented with 25 mg cystine per animal per day. Accurate records of food consumption were kept, so it was possible to calculate (1) the protein efficiency ratios, expressed as gain in weight per gram of protein intake; (2) the biological values, expressed as the per cent of absorbed nitrogen retained by the rat; and (3) the gain in body weight per 100 gm of food intake, which expressed the requirements for maintenance and growth and was the yardstick we used for evaluating economy of food utilization.

Before beginning the nitrogen balance studies, the rats were depleted of the vitamin B complex for 10 to 15 days until either maintenance was reached or there were slight losses in body weight. In starting the metabolism experiments the animals were 25 to 28 days of age and weighed about 50 gm.

In the construction of the vitamin B complex synthetic mixtures we used for the daily doses of thiamine, riboflavin, pyridoxine, and niacin, 3 to 100 μg; for calcium pantothenate, 25 to 600 μg; for *p*-aminobenzoic acid, 250 μg to 12 mg; for choline chloride, 3 to 12 mg; and for inositol, 75 μg to 3 mg (table 1). These mixtures (VBC)[3] are hereinafter referred to either as the numbers 1 to 7 given them in table 1 or as mixtures having the 3, 5, 10, and 15 μg doses, since the same daily doses were used for 4 components of the vitamin B complex.

## RESULTS

Data from these experiments are submitted in tables 2, 3, and 4. It will be noted from table 2 that, on a 20% casein intake, increasing various components of the vitamin B complex produced no notable changes in nitrogen retention during

[3] VBC = vitamin B complex.

TABLE 2

*Influence of concentration of the vitamin B complex (VBC) on biological values of protein and on economy of food utilization*

*(Average results for 5 animals in each group, 3 ♀'s and 2 ♂'s)*

| PROTEIN | VBC MIXTURES [1] | DAILY DOSES OF THIAMINE, RIBOFLAVIN AND NIACIN | GAIN IN BODY WEIGHT | TOTAL FOOD INTAKE | INCREASE IN FOOD INTAKE | GAIN IN BODY WEIGHT PER 100 GM FOOD INTAKE | INCREASE IN FOOD UTILIZATION | PROTEIN EFFICIENCY RATIO [2] | BIOLOGICAL VALUE [3] |
|---|---|---|---|---|---|---|---|---|---|
| | | *μg* | *gm* | *gm* | % | *gm* | % | | |
| 20% | 1 | 3 | 6.6 | 32.3 | ... | 20.4 | ... | 1.19 ± 0.15 [5] | 61.6 ± 4.66 [6] |
| Casein [4] | 2 | 5 | 10.0 | 38.2 | 18.3 | 26.2 | 28.4 | 1.64 ± 0.18 | 62.6 ± 2.52 |
| (17.3% | 3 | 10 | 21.2 | 46.8 | 44.9 | 45.3 | 122.1 | 2.61 ± 0.07 | 63.2 ± 2.00 |
| protein) | 4 | 15 | 16.6 | 42.9 | 32.8 | 38.7 | 89.7 | 2.44 ± 0.06 | 59.0 ± 6.45 |
| 10% | | | | | | | | | |
| Casein [4] | 1 | 3 | 3.8 | 31.5 | ... | 12.1 | ... | 1.63 ± 0.23 | 71.8 ± 3.55 |
| (8.7% pro- | 2 | 5 | 9.2 | 37.1 | 17.8 | 24.8 | 105.0 | 2.87 ± 0.17 | 79.0 ± 2.19 |
| tein) + | 3 | 10 | 18.2 | 47.7 | 51.4 | 38.2 | 215.7 | 4.40 ± 0.07 | 77.5 ± 3.35 |
| 25 mg | 4 | 15 | 22.8 | 53.6 | 71.4 | 42.5 | 251.2 | 4.79 ± 0.31 | 77.7 ± 3.87 |
| cystine/ rat/day | | | | | | | | | |

[1] See table 1.

[2] Gain in body weight per gram of protein intake.

[3] Per cent of absorbed nitrogen retained in the body.

[4] Smaco.

[5] Standard deviation of the means.

[6] Standard deviation (Sherman, '41).

the 7-day metabolism period. However, a change from VBC mixture 1 to 3 resulted in an increase of about 50% in food intake and of over three times the gain in body weight. The increase of 119% in the so-called "protein efficiency ratio" parallels the increase of 122% in total food utilization. On a 10% plane of casein intake, increasing the daily doses of thiamine, riboflavin, pyridoxine, and niacin from 3 to 15 μg, with the same increased doses of the rest of the components of the vitamin B complex mixtures, also produced no significant changes in nitrogen retention during the one-week balance study. On the other hand, there was a 70% increase in food intake and a 6-fold gain in body weight, which establish conclusively that the enhanced growth following the increases in the concentration of the vitamin B complex was entirely out of proportion to the rise in food consumption and the marked gains in food utilization should be ascribed to the greater intake of the B vitamins. The increase from the 3 to 15 μg doses produced a rise of from 1.63 to 4.79, or 193%, in the so-called "protein efficiency ratio," and an increase of 251% in total food utilization.

On an 8.2% level of casein intake, introducing 7.1% protein in the ration, the influence on growth and food utilization was most marked. The increase from the 3 to the 5 μg daily doses (table 3), produced a marked increase in nitrogen retention. This is undoubtedly due to the increased caloric intake associated with the increased concentration of the vitamin B complex mixture.

The high figure for the standard deviation of the biological value on VBC mixture 1 during the first metabolism period (table 3) is due to the great variations in food intake and to variations in the small increments of growth on this low mixture of the vitamin B complex.

Following a standardizing period, the animals which were receiving VBC mixtures 1, 2, 3, and 4 were given, respectively, VBC mixtures 4, 5, 6, and 7, thus increasing the daily doses of thiamine, riboflavin, pyridoxine, and niacin from 3, 5, 10, and 15, to 15, 25, 50, and 100 μg. This shift to higher doses of

TABLE 3

*Influence of concentration of the vitamin B complex (VBC) on biological values of protein and on economy of food utilization*

*(Average results for 4 animals in each group, 2 ♀'s and 2 ♂'s)*

| PROTEIN | VBC MIXTURES [1] | DAILY DOSES OF THIAMINE, RIBOFLAVIN AND NIACIN | GAIN IN BODY WEIGHT | TOTAL FOOD INTAKE | INCREASE IN FOOD INTAKE | GAIN IN BODY WEIGHT PER 100 GM FOOD INTAKE | INCREASE IN FOOD UTILIZATION | PROTEIN EFFICIENCY RATIO [2] | BIOLOGICAL VALUE [3] |
|---|---|---|---|---|---|---|---|---|---|
| | | *γγ* | *gm* | *gm* | % | *gm* | % | | |
| 8.2% Casein [4] (7.1% protein) + 25 mg cystine/ rat/day | 1 | 3 | 0.75 | 26.8 | ... | 2.8 | ..... | 0.68 ± .17 [5] | 46.3 ± 8.4 [6] |
| | 2 | 5 | 6.5 | 37.8 | 41.1 | 17.2 | 514.3 | 2.37 ± .62 | 81.6 ± 3.5 |
| | 3 | 10 | 17.2 | 48.1 | 79.5 | 35.8 | 1178.6 | 4.94 ± .63 | 85.2 ± 2.7 |
| | 4 | 15 | 22.2 | 57.4 | 114.2 | 30.7 | 1282.2 | 5.51 ± .44 | 85.0 ± 1.3 |
| | | | | | Second metabolism periods [7] | | | | |
| | 4 | 15 | 17.0 | 61.6 | ... | 27.6 | ... | 3.88 ± .36 | 72.7 ± 3.66 |
| | 5 | 25 | 23.0 | 73.8 | 19.8 | 31.2 | 13.0 | 4.42 ± .39 | 69.5 ± 4.67 |
| | 6 | 50 | 27.0 | 80.8 | 31.2 | 33.4 | 21.0 | 4.73 ± .22 | 70.2 ± 2.64 |
| | 7 | 100 | 23.2 | 76.5 | 24.2 | 30.3 | 9.8 | 4.28 ± .35 | 64.3 ± 3.56 |

[1] See table 1.

[2] Gain in body weight per gram of protein intake.

[3] Per cent of absorbed nitrogen retained in the body.

[4] Smaco.

[5] Standard deviation of the means.

[6] Standard deviation (Sherman, '41).

[7] Following a standardizing period, the animals which received vitamin B mixtures 1, 2, 3, 4, were given, respectively, vitamin B mixtures 4, 5, 6, and 7 during the second one-week metabolism period.

the vitamin B complex, even on the lower level of protein intake (7.1%) resulted in no change in efficiency of nitrogen retention, but this change in vitamin concentration was accompanied by a marked increase in body weight and in food consumption. Optimum results were obtained on VBC mixture no. 6. A further increase in the vitamin B complex, from this mixture to no. 7, produced a depression in growth and in efficiency of protein utilization. These results may have practical applications in the war-torn countries abroad where pronounced food shortages still exist. On the basis of data presented in table 3 it appears that on a low protein level (7.1%) some growth was possible but only when the various components of the vitamin B complex were raised to high concentrations. On the same low protein intake and with a low concentration of the B vitamins, only maintenance was obtained. These observations were made during a one-week experimental period. During longer periods on the low vitamin B complex and low protein diet, these animals, undoubtedly, would have collapsed. At least, experiences in this laboratory make this seem probable.

A study was also carried out on the influence of concentration of the vitamin B complex on the chemical composition of body gains. Twelve male and 12 female rats were used for this investigation. They were a month old and weighed 50 to 59 gm each when started on experiment. They were depleted of the vitamin B complex for 12 to 15 days, were placed on a preliminary vitamin period for several days to get accustomed to the new dietary regimen, and then were kept for 21 days on a 10% casein diet with graduated increasing doses of VBC mixtures 1, 2, 3, and 4. In addition, 12 male and 12 female rats of the same initial age and weight were taken one week after weaning from stock diets and analyzed for moisture, protein, fat, and ash. From the chemical compositions and body weights at the beginning and at the end of the experimental periods the chemical composition of body gains was computed. The results are submitted in table 4. It will be noted that during this three-week period a change from

VBC mixture 1 to 2, increasing from the 3 to the 5 μg doses, resulted in increased protein synthesis, with no further increased protein synthesis following further increases of vitamin B complex concentrations. However, synthesis of fat resulted from the further change from VBC mixture 2 to 3, when the daily doses were increased from 5 to 10 μg. The marked increases in growth following increase in certain concentrations of the vitamin B complex must then be due to fat synthesis from the carbohydrate (cerelose) in the rations. The only notable change resulting from raising the daily doses from 10 to 15 μg was in the ash content; we have no explanation for the reduction in ash when the VBC mixture was changed from 2 to 3, increasing the daily doses from 5 to 10 μg.

The fact that we observed in this investigation frequent parallelisms between per cent increase in food utilization and per cent increase in protein efficiency ratios and, in most cases, no parallelism between biological values (as determined from nitrogen balance studies) and protein efficiency ratios, would indicate that the latter do not always express solely the gains in weight per gram of protein intake. The so-called "protein efficiency ratios" must also include gains in body weight pro-

TABLE 4

*Influence of concentration of the vitamin B complex (VBC) on chemical composition of body gains*[1] *(Average results for 6 animals in each group, 3 ♀'s and 3 ♂'s)*

| VBC MIXTURES[1] | DAILY DOSES OF THIAMINE, RIBOFLAVIN AND NIACIN | GAIN IN BODY WEIGHT[2] | INCREASE IN BODY WEIGHT | GAIN IN BODY WEIGHT PER 100 GM FOOD INTAKE | INCREASE IN FOOD UTILIZATION | PROTEIN EFFICIENCY RATIO | CHEMICAL COMPOSITION OF BODY GAINS | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Ash | Fat | Protein |
| | μg | gm | % | gm | % | | gm | gm | gm |
| 1 | 3 | 22.0 | ... | 16.7 | ... | 1.95 ± .16[3] | + 1.69 | + 0.93 | + 5.06 |
| 2 | 5 | 29.0 | 31.8 | 18.5 | 10.8 | 2.15 ± .10 | + 2.15 | + 3.70 | + 8.65 |
| 3 | 10 | 41.0 | 86.4 | 22.3 | 33.5 | 2.58 ± .10 | + 1.89 | + 6.26 | + 8.75 |
| 4 | 15 | 41.7 | 80.6 | 22.0 | 31.7 | 2.54 ± .07 | + 2.47 | + 5.81 | + 9.09 |

[1] See table 1.

[2] Experimental period of 21 days. The animals received a 10% casein diet, supplemented with 25 mg cystine per animal per day.

[3] Standard deviation of the means (Sherman, '41).

duced by caloric intakes from the ration. Our observations are in agreement with those of Barnes and Bosshardt ('46) who state that ". . . the influence of caloric intake upon the growth utilization of protein may be greater than has been suspected, heretofore." Bosshardt and associates ('46b) in their recent protein studies with mice fed ad libitum found that ". . . with each protein source the level of intake exhibiting maximal protein utilization corresponded with a maximal caloric intake per unit of body surface area, and at a given level of protein intake changes in caloric consumption often resulted in the apparent utilization of protein."

A study was also made of the influence of aqueous butyl alcohol extracts of Wilson's 1:20 liver concentrate powder on the utilization of casein for growth. The casein was fed at a 10% plane of intake and the extracts of the liver concentrate were prepared by the procedure of Conger and Elvehjem ('41). A total of 48 rats was used in this investigation. The extracts were fed at 0.5 and 0.2% of the rations. It was anticipated that such extracts might furnish unknown components of the vitamin B complex. Following 11 weeks' growth, the results were entirely negative. Such extracts also resulted in no increases in nitrogen retention, as evidenced by balance studies. Our observations on rats are, therefore, not in accord with those of Bosshardt and co-workers on mice ('46a).

## SUMMARY

Increasing the concentrations of thiamine, riboflavin, pyridoxine, niacin, pantothenic acid, choline, *p*-aminobenzoic acid, and inositol used in synthetic mixtures as sources of the vitamin B complex resulted in marked increases in growth and pronounced increases in food utilization. However, increases in nitrogen retention and protein synthesis occurred only within a narrow range of the low concentration of the vitamin B complex. The marked increases in growth are due largely to fat synthesis from the carbohydrate (cerelose) in the rations.

On a low 7.1% protein level some growth was possible, but only when the various components of the vitamin B complex were raised to high concentrations. On the same low protein intake and on a low concentration of the B vitamins, only maintenance was obtained. These observations were made during a one-week experimental period.

The results of feeding aqueous butyl alcohol extracts of 1:20 Wilson's concentrate powder as a source of unknown components of the vitamin B complex were negative as far as utilization of casein for growth is concerned.

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# ANEMIA AND EDEMA OF CHRONIC CHOLINE DEFICIENCY IN THE RAT[1]

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FOUR FIGURES

(Received for publication July 19, 1948)

For some years this laboratory has conducted investigations to determine the effects of choline deficiency in the rat. During these studies it was observed that neoplasms develop in the livers and other tissues in a significant number of animals subjected to such a deficiency for prolonged periods (Copeland and Salmon, '46; Engel, Copeland and Salmon, '47). It seemed desirable to determine if some physiological abnormality might appear in such animals during the develment of the chronic choline deficiency syndrome. A lowered hemoglobin has been reported in dogs fed choline-deficient diets (Fouts, '43; McKibbin et al., '44). Attention was therefore directed to the blood; hemoglobin determinations were made on rats in an experiment in which a variety of choline-low and/or protein-low diets were under investigation.

The production of anemia as well as nutritional edema as a result of choline deficiency will be discussed in the following pages.

## EXPERIMENTAL

Rats of the Alabama Experiment Station (AES) strain were used. The animals were fed the experimental diets from

[1] Published with the approval of the Director, Alabama Agricultural Experiment Station, Auburn. A preliminary report of this work was made at the annual meeting of the Federation of American Societies for Experimental Biology, Federation Proceedings, Volume 7, Number 1, Part 1, p. 285, 1948.

the age of 23 days, except in one case in which older animals were used. Care was exercised to maintain litter and sex controls in studying treatment effects. The animals were housed individually in screened-bottom cages, and were supplied with fresh food daily.

The percentage composition of the diets used in these studies is given in table 1. The diets were stored in a refrigerator and were replenished about once every 10 days.

TABLE 1

*Percentage composition of the basal diets* [1]

| INGREDIENT | DIET NO. | | | | | | |
|---|---|---|---|---|---|---|---|
| | 46E | 57 | C-1 | 46 | 59 | 58 | 60 |
| Extracted casein [2] | 4.5 | 6.5 | 9.0 | 9.0 | 9.0 | 0.0 | 6.0 |
| Extracted peanut meal [3] | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 30.0 | 30.0 |
| Degerminated corn grits [4] | 40.0 | 40.0 | 20.0 | 40.0 | 30.0 | 30.0 | 0.0 |
| Salts [5] | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Sucrose | 36.2 | 34.2 | 51.7 | 31.7 | 36.7 | 15.9 | 39.9 |
| Lard | 14.0 | 14.0 | 14.0 | 14.0 | 19.0 | 19.0 | 19.0 |
| Cod liver oil | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| L-cystine | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 | 0.1 | 0.1 |

[1] Each kilogram of basal diet was fortified with the following: 2 mg each of thiamine and pyridoxine, 4 mg of riboflavin, 10 mg of calcium pantothenate, 20 mg of niacin, 200 mg of i-inositol and 50 mg of alpha-tocopherol. Control animals received a further supplement of 2000 mg of choline chloride per kilogram of diet. The author is indebted to Merck and Company, Rahway, New Jersey, for a supply of these vitamins.

[2] Commercial casein was purified by percolation with tap water for 6 days with overnight acidifications (0.2% acetic acid) and finally washed with 95% alcohol and dried. The purified product contained 87% protein (N × 6.38).

[3] Commercial peanut meal was percolated exhaustively with water solution containing 60% ethanol by volume and finally extracted with absolute methanol and dried. The extracted material contained 44% crude protein (N × 6.25).

[4] A non-enriched commercial product containing 8.8% crude protein (N × 6.25).

[5] J. Nutrition, *33:* 155–168, 1947.

### *Effect of choline or methionine on hemoglobin*

Hemoglobin determinations were made at least once monthly. The samples were obtained by bleeding from the tail. The acid hematin method was used, and the color den-

sity was measured with filter 420 in an Evelyn photoelectric colorimeter. The instrument was calibrated with graded concentrations of pure hematin prepared from beef blood according to the method of Clifcorn and associates ('35).

The results of these analyses are summarized in table 2. A lowered hemoglobin with a range of 6.25 to 11.95 gm per 100 ml blood was consistently observed in animals maintained on the choline-deficient diets for three months or longer.

TABLE 2

*Final hemoglobin concentration and body weight of rats fed diets varying in protein and fat with and without supplement of choline or methionine (6-month experimental period)*

| NO. OF ANIMALS PER TREATMENT | SEX | DIET NO. | TREATMENT | | | | DIETARY PROTEIN | DIETARY FAT |
|---|---|---|---|---|---|---|---|---|
| | | | Choline added | | No choline added | | | |
| | | | Body wt. | Hemoglobin | Body wt. | Hemoglobin | | |
| | | | *gm* | *gm/100 ml blood* | *gm* | *gm/100 ml blood* | % | % |
| 2 | M | 46E [1] | 546 | 13.60 | 339 | 6.25 | 7.5 | 15 |
| 3 | F | 46E [2] | 339 | 14.20 | 335 | 6.85 | 7.5 | 15 |
| 6 | M | 57 | 388 | 13.45 | 353 | 8.75 | 9.2 | 15 |
| 2 | F | 57 | 271 | 13.85 | 317 | 10.05 | 9.2 | 15 |
| 3 | M | C-1 | 438 | 14.40 | 320 | 8.55 | 9.6 | 15 |
| 2 | F | C-1 | 265 | 14.25 | 283 | 11.80 | 9.6 | 15 |
| 2 | M | C-1 [3] | 445 | 14.25 | 444 | 14.90 | 9.9 | 15 |
| 2 | F | C-1 [3] | 277 | 13.85 | 260 | 14.55 | 9.9 | 15 |
| 2 | M | 46 | 472 | 13.00 | 403 | 10.10 | 11.4 | 15 |
| 2 | F | 46 | 287 | 13.85 | 320 | 11.95 | 11.4 | 15 |
| 2 | M | 46 [3] | 515 | 12.20 | 451 | 13.75 | 11.7 | 15 |
| 2 | F | 46 [3] | 303 | 14.55 | 339 | 14.70 | 11.7 | 15 |
| 2 | M | 59 | 459 | 14.30 | 284 | 7.85 | 10.5 | 20 |
| 2 | M | 58 | 404 | 13.80 | 202 | 9.85 | 15.8 | 20 |
| 2 | F | 58 | 223 | 14.65 | 178 | 10.05 | 15.8 | 20 |
| 2 | M | 60 | 431 | 14.30 | 309 | 10.30 | 19.2 | 20 |

[1] These animals were placed on diet 46E after they had received diet 46 for 6 months after weaning.

[2] These animals were placed on diet 46E after 9–12 months on a normal stock diet.

[3] These animals received a supplement of 3 gm of DL-methionine per kilogram of diet.

Male animals generally became slightly more anemic and made poorer weight gains on the deficient diets than females. The severity of the anemia appeared to be correlated with the protein content of the diet, the lowest hemoglobin levels occurring in the animals receiving the diets most deficient in protein. The important finding, however, is that the anemia was prevented by choline or methionine even at the lowest protein levels.

The anemia developed within the first three months after the animals were placed on the diets unsupplemented with choline or methionine. The hemoglobin concentration remained essentially unchanged at the lowered levels between

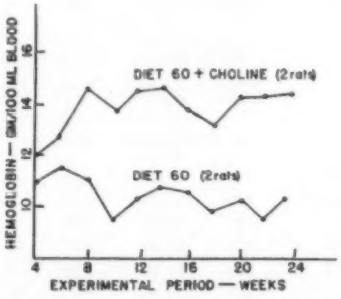


Fig. 1 Average hemoglobin concentration at 2-week intervals in rats fed diets with and without added choline.

the third and 6th months. In one study, hemoglobin determinations were made at two-week intervals between the 4th and the 24th week in order to follow more closely the development of the anemia. The results are presented in figure 1. Since rats of this strain do not survive if dietary choline is not supplied during the period of rapid growth, it was our practice to supply animals on the choline-deficient diets with enough choline to prevent fatal kidney damage during the first month of the experiment. In the case of the choline-deficient animals represented in figure 1 this requirement was met by supplementing the diet with 650 mg of choline chloride per kilogram. As can be seen in this figure, the

choline-deficient animals became anemic rather rapidly between the 6th and 10th weeks of the experiment. The lowered hemoglobin levels remained more or less unchanged during the subsequent 14-week period.

### *Ineffectiveness of folic acid in curing the choline-deficiency anemia*

Since the diets did not contain a supplementary source of folic acid, it seemed desirable to determine the effectiveness of this factor in the anemia that results from lack of choline. For this purpose animals that had developed the anemic condition while subsisting on choline-deficient diet 57 for 4 months were given a supplement of 2 mg of the sodium salt of pteroyl-diglutamic acid [2] per kilogram of diet. There was no improvement during the subsequent 4-month peri d. Hemoglobin levels remained unchanged and were comparable to those observed in litter mates not receiving the supplement.

### *Edema in chronic choline deficiency*

The animals receiving the choline-deficient diet lowest in protein, Diet 46E, became more anemic than did animals on diets less restricted in protein. It was observed that a marked accumulation of fluid occurred in the abdominal and thoracic cavities as well as in the subcutaneous tissues of some of the animals receiving Diet 46E without choline supplement. To date this marked edema has been observed in 7 of 12 animals receiving this diet for 4 months or longer. One case of severe edema has been observed in an animal receiving choline-deficient Diet 57, a diet similar to Diet 46E except that it contained 6.5% casein instead of 4.5%. This edematous condition has not been observed in animals receiving the same diets supplemented with choline.

The severity of this edema is illustrated in the photographs in plate 1. The edema was consistently accompanied by severe

[2] The Laboratory is indebted to Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, for a supply of this compound.

hepatic cirrhosis evidenced grossly by an irregular, nodular liver surface.

## DISCUSSION

It is possible that the function of choline in preventing anemia in these experiments is that of sparing an amino acid essential for the synthesis of hemoglobin protein; namely, methionine. The effectiveness of DL-methionine in preventing anemia in the present study supports this hypothesis. Robscheit-Robbins and associates ('47) have demonstrated an increased output of blood proteins in protein-depleted dogs by the addition of methionine to an otherwise complete amino acid mixture. Albanese and co-workers ('46) observed a reduction in hemoglobin in rats fed diets nearly devoid of methionine. Li and Freeman ('47) fed rats a diet containing 9% casein as the source of protein and observed a marked growth stimulation and a slight rise in hemoglobin when the diet was supplemented with methionine. These investigators relied upon brewers' yeast at a level of 0.5 gm per rat per day as the source of choline. The adequacy of choline intake could be questioned in such a diet unsupplemented with methionine, since that amount of yeast would supply only about 2.5 mg of choline per rat per day (Engel, '42). The effectiveness of choline in preventing the severe anemia which occurred on diets containing as low as 4.5 or 6.5% casein in the present study would suggest either a striking protein-sparing property of this nutrient or some still unknown relationship between it and hematopoiesis.

The possibility must be considered that the severely damaged liver resulting from chronic choline deficiency may in some way interfere with normal hemoglobin or red blood cell formation. Moosnick and associates ('45) have made the interesting observation that choline may have therapeutic value in certain human anemias. They treated a pernicious anemia patient refractory to purified liver extract with intravenous choline and noted a definite response. Prior to treatment the patient showed fatty metamorphosis of the liver. Davis and Brown ('47) have recently reported on the use of

choline in megaloblastic anemias. They observed a significant response to choline in two cases resembling Addisonian pernicious anemia which were refractory to parenteral liver extracts.

It is possible that there may be a relationship between the lowered hemoglobin and the development of neoplasms in rats subjected to a chronic choline deficiency. Other investigators, in studying pre-cancerous changes in animals, have noted a correlation between tumor induction and hemoglobin level. Strong and Frances ('40) observed a gradual drop in hemoglobin in a highly tumor-susceptible strain of mice during the pre-cancerous stages, an abnormality which did not occur in mice of a strain relatively resistant to mammary tumor development. Taylor and Pollack ('42) likewise reported lowered hemoglobin during the pre-cancerous stage in mice injected with methylcholanthrene and also in rats fed diets containing *p*-dimethylaminoazobenzene.

The occurrence of prominent symptoms of nutritional edema with severe ascites in rats fed low-protein diets unsupplemented with choline raises a question as to the possible importance of choline in nutritional edema in the human. This disease is usually associated with an inadequate protein intake. Under such conditions the choline intake likewise would probably be inadequate. It is significant that in the present study the edema was consistently accompanied by severe liver damage, a condition which is not uncommon in human nutritional edema. The method reported here for the experimental production of nutritional edema should be valuable for carefully examining the therapeutic value of choline or related nutrients in the control of this disease.

## SUMMARY

Prolonged feeding of diets deficient in choline produced an anemia in rats. The hemoglobin levels in the deficient animals ranged from 6.25 to 11.95 gm per 100 ml of blood. The anemia was prevented by dietary supplements of choline

chloride or DL-methionine. The sodium salt pteroyl-diglutamic acid was ineffective as a curative agent.

The prolonged feeding of diets low in protein and choline resulted in symptoms of severe nutritional edema in 7 of 12 rats. This condition was not observed in control animals receiving the same diets supplemented with choline.

ACKNOWLEDGMENT

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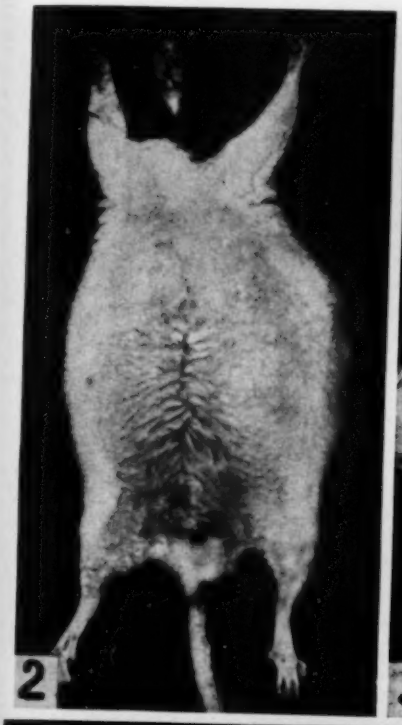
## PLATE 1

### EXPLANATION OF FIGURES

2 Gross nutritional edema with marked ascites in a rat which received Diet 57 without choline for 11 months.

3 Same rat with a superficial dissection to illustrate fluid accumulation throughout the subcutaneous tissues and extensive ascites as evidenced by a severely distended abdomen.

4 Same rat with thoracic and abdominal cavities exposed. At autopsy 169 gm of a clear, slightly yellow fluid was drained from the abdominal cavity of this animal which weighed 380 gm at death. Note the cirrhotic condition of the liver and the severely atrophied testes. There is a complete absence of visceral fat stores in this condition.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# GROWTH EFFICIENCY OF ESSENTIAL AMINO ACIDS ALONE AND IN COMBINATION WITH CASEIN [1,2]

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ONE FIGURE

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The well-known report of Rose ('37) concerning the indispensability of certain amino acids for growth of the white rat, and his estimation of minimum requirements, have given rise to a number of studies in this field, some of which will be referred to in the discussion of the results of the present experiment.

The objectives of the present study were: (1) to test again the growth-promoting value of the 10 essential amino acids when fed as the sole source of nitrogen, at the levels suggested by Rose; (2) to determine the effectiveness of the essential amino acids when fed in part as casein and in part in the crystalline form; and (3) to determine the effects of feeding the essential amino acids, as the sole source of nitrogen, at levels higher than the suggested minimum.

## EXPERIMENTAL

Diets of the purified type were used and the ingredients common to all diets were the following: corn oil, 9%; cod

[1] Journal Series paper of the Department of Agricultural Biochemistry, New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick.

[2] Presented before the Division of Biological Chemistry, American Chemical Society, New York, N. Y., September, 1947.

liver oil, 1%; sodium chloride, 1%; and salt mixture, U. S. P. No. 1, 4%. Crystalline amino acids were ground with cane sugar to make 10% or 20% of the diet, along with the quantity of sodium bicarbonate necessary to neutralize the hydrochloric acid of arginine, histidine and lysine hydrochlorides. Casein was used in the amounts indicated in table 1, and the balance of each diet consisted of corn starch. Water soluble vitamins and other organic factors were added in the following quantities per 100 gm of diet: thiamine, 0.5 mg; riboflavin, 1.0 mg; pyridoxine, 0.5 mg; calcium pantothenate, 2.5 mg; nicotinic acid, 2.0 mg; inositol, 0.25 mg; *p*-aminobenzoic acid, 5.0 mg; choline, 20 mg; biotin, 2 μg; and folic acid, 0.1 mg.

Each test group consisted of 2 male and 2 female white rats, 21–24 days of age and weighing between 55 and 65 gm. The animals were housed in individual cages and fed ad libitum. The casein [3] used in the experiment had been analyzed microbiologically by Stokes and associates ('45). For diet A (table 1) the quantities of the crystalline amino acids fed were the minimum levels suggested by Rose ('37), except that the quantity of leucine was reduced from 900 to 800 mg and of threonine from 600 to 500 mg per 100 gm of diet.[4]

## RESULTS AND DISCUSSION

Table 1 shows the source of nitrogen fed each group, the nitrogen distribution in terms of the essential and non-essential nitrogen of casein and of the active and inactive isomers of the crystalline amino acids, the growth response, and the food consumption.

It is of interest that 50% of the nitrogen of the casein is that of essential amino acids and 50% is non-essential in character. The increase in percentage of casein is accompanied by an increase in non-essential nitrogen from that source and by a decrease in the quantity of inactive crystalline D-isomers. Although with the increase in casein content there is an in-

[3] SMA Vitamin Test Casein. Lot No. 14126.

[4] In accordance with suggestions made in a personal communication from Dr. W. C. Rose.

TABLE 1

*Nitrogen per 100 gm of diet, protein equivalents, growth response and food consumption (20-day period)*

| DIET | CASEIN [1] | | CRYSTALLINE AMINO ACIDS | | TOTAL N | TOTAL ESSENTIAL N | TOTAL N MINUS INACTIVE D-ISOMERS | PROTEIN EQUIVALENT (N×6.25)[2] | AVERAGE DAILY GAIN (20-DAY PERIOD) | AV. DAILY FOOD INTAKE | GAIN PER GM OF PROTEIN EQUIVALENT (N×6.25) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Essential N | Non-essential N | Essential N | N of inactive D-isomers | | | | | | | |
| | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* | *mg* | % | *gm* | *gm* | *gm* |
| A — Crystalline amino acids | ... | ... | 788 | 195 | 983 | 788 | 788 | 4.93 | 0.8 | 7.3 | 2.2 |
| B — 2% Casein [3] + A.A. | 147 | 146 | 641 | 158 | 1092 | 788 | 934 | 5.84 | 1.1 | 7.6 | 2.5 |
| C — 4% Casein + A.A. | 293 | 293 | 498 | 117 | 1201 | 791 | 1084 | 6.78 | 1.7 | 7.2 | 3.5 |
| D — 6% Casein + A.A. | 440 | 439 | 360 | 82 | 1322 | 800 | 1240 | 7.76 | 2.4 | 8.3 | 3.8 |
| E — 8% Casein + A.A. | 586 | 586 | 229 | 54 | 1455 | 815 | 1400 | 8.78 | 3.9 | 9.5 | 4.7 |
| F — 1.28 × A.A. | ... | ... | 1010 | 248 | 1258 | 1010 | 1010 | 6.32 | 1.2 | 6.7 | 2.8 |
| G — 1.77 × A.A. | ... | ... | 1400 | 342 | 1742 | 1400 | 1400 | 8.78 | 1.2 | 4.6 | 3.0 |

[1] N content of casein 13.63%, air-dried basis.

[2] Total nitrogen minus nitrogen of inactive D-isomers × 6.25.

[3] Calculated to dry basis.

crease in total nitrogen minus the inactive D-isomers of the crystalline acids, the quantity of essential nitrogen in the casein-containing diets was kept constant at the quantity provided by the minimum suggested levels of the essential amino acids, except for a slight excess at the 6% and 8% casein levels due to the arginine nitrogen supplied by casein. An increase in the quantity of the essential amino acids, when they were the sole source of nitrogen in the diet, provided a higher level of essential nitrogen. The protein equivalents ($N \times 6.25$) range from 4.93% for the diet containing the mini-

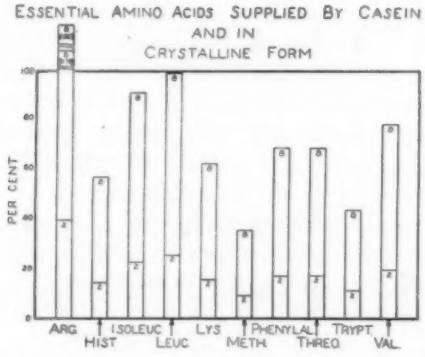


**Fig. 1** For each amino acid the 100% line represents the requirements suggested by Rose ('37). The bars show the percentages of the amino acids supplied by the 2% and 8% levels of casein, the 4% and 6% levels being proportionately intermediate. The percentage of a crystalline acid is represented by the distance from the top of a bar to the 100% line.

mum amino acid levels to 8.78% for that made with 8% casein and added amino acids and that designed to provide 1.77 times the minimum amino acid requirement.

Figure 1 shows the percentages of the minimum levels of the essential amino acids supplied as casein and in the crystalline form. Except for arginine, none of the essential amino acids was supplied by casein in excess of the minimum requirement. When the level of casein was 8%, it was necessary to add only 0.8% of the leucine requirement as the crystalline acid, but for the other essential acids additions of the crys-

talline form ranged from 10.4% of the requirement for isoleucine to 65.2% of that for methionine. Higher levels of casein were not used, since the levels of several amino acids would have exceeded the minimum requirements.

When the minimum quantity of essential amino acids suggested as necessary for growth was fed (diet A), the average daily gain was 0.8 gm, which is what might be expected since the nitrogen of the diet is equivalent to only 4.93% protein. This response is slightly more than, but of the same order as, that reported by Kinsey and Grant ('44). It is not, however, in agreement with the report of Albanese and Irby ('43), who failed to obtain growth even when more than the minimum quantities of the essential amino acids were fed. An explanation of this difference in results has not been found, but the question may be raised as to whether the isoleucine content of the diet used by Albanese and Irby was adequate, since this amino acid was supplied as a mixture of leucine and isoleucine, leucine being provided also as a single amino acid.

At the 2% casein level the daily gain of 1.1 gm was not much better than that resulting when the crystalline acids were the sole source of nitrogen, but at 4%, 6% and 8% casein levels the growth responses were significantly greater and increased progressively with the increase in casein content until an average daily gain of 3.9 gm was obtained with diet E containing 8% casein. This is a very satisfactory rate of gain when it is considered that the diet contained nitrogen equivalent to only 8.78% protein, a quantity which would be equal in nitrogen content to 10.3% casein. The response of 3.9 gm is in close agreement with that of 3.4 gm obtained by Womack and Rose ('46) when they fed a mixture of 10 essential and 9 non-essential amino acids, although these authors did not report the quantities of amino acids used.

The progressive increase in daily gain is associated with an increase in non-essential nitrogen and a decrease in the quantity of inactive D-isomers, the quantity of essential nitrogen remaining practically constant. Whether the better gains

are due entirely to the nitrogen provided by the greater quantities of non-essential amino acids, or to this type of nitrogen along with some essential factor such as strepogenin (Woolley, '46), is as yet unknown. The close agreement with the response reported by Womack and Rose ('46) when crystalline acids were the sole source of nitrogen suggests, at least, that it is the nitrogen of the non-essential acids, rather than some essential factor, that is responsible for the gains shown on diet E. The question of whether certain of the non-essential amino acids are more necessary than others for the full effectiveness of the essential acids in promoting growth is one that has not yet been answered. Kinsey and Grant ('44) found, for example, that the addition of glycine to a diet containing the essential acids at the minimum required level failed to improve the growth rate, and Hier, Graham and Klein ('44) noted an inhibition of growth when glycine and L-proline were added separately to casein and fibrin diets.

It was necessary to use DL forms of three amino acids — isoleucine, threonine, and valine — whose D forms according to Rose ('38) are inactive for the rat. In diet A, the contents of inactive D-isomers per 100 gm of diet were 500 mg of isoleucine, 500 mg of threonine and 700 mg of valine. With the increase in the percentage of casein, the quantities of inactive isomers decreased until at the 8% level of casein the content of D-isoleucine became 52 mg per 100 gm of diet, of D-threonine 164 mg, and of D-valine 164 mg. Whether the inactive D-isomers have a retarding effect on growth, and their progressive decrease in the casein-amino diets allowed better growth, is not known. It should be noted, however, that for diets F and G, which contained the largest quantities of inactive isomers, growth was not depressed below that of diet A, which contained the next highest levels of these isomers. Thus, for diet F, the quantities of the inactive D-isomers of isoleucine, threonine, and valine per 100 gm of diet were 640 mg, 640 mg, and 896 mg, respectively, whereas for diet G the quantities were 885 mg, 885 mg, and 1239 mg.

The question of whether an excess of the essential amino acids, fed as the sole source of nitrogen, over the suggested minimum levels will supply the additional nitrogen necessary to promote better growth was given attention in the present investigation. Diet F, which contained 1.28 times the minimum suggested quantities of the essential amino acids, provided 1010 mg of essential nitrogen per 100 gm of diet, a value which falls between the total nitrogen minus the nitrogen of the inactive D-isomers of the 2% and 4% casein diets. Despite the fact that the 1010 mg of essential nitrogen is 28% greater than the 790 mg of these latter diets, the growth response is the same as that for the 2% casein diet. The factor 1.77 was used to provide a diet in which the total utilizable nitrogen would be 1400 mg per 100 gm of diet, the same as that in diet E which contained 8% casein and crystalline amino acids, and equivalent to 8.78% protein. Even with this increase in utilizable essential nitrogen the average daily gain was only 1.2 gm, which is no better than that noted when 2% casein and the essential acids (or 1.28 times the minimum amino acid levels) was the source of nitrogen. Similar observations have been made by Martin ('44), by Kinsey and Grant ('44) and by Hier, Graham and Klein ('44).

When the quantity of essential nitrogen was 815 mg and of non-essential 586 mg (diet E), excellent growth occurred. Diet F supplied 815 mg of essential nitrogen, which should meet the minimum requirements for good growth, and 585 mg of additional essential nitrogen in place of the non-essential nitrogen of diet E, yet poor growth resulted. Why this additional essential nitrogen is not utilized by the rat is a question of considerable interest.

The daily food consumption showed a progressive increase from 7.3 gm per rat per day for the animals on diet A to 9.5 gm for those on diet E, except in the case of diet C for which the value is not as high as would be expected. The increase in food consumption was accompanied by an increase in average daily gain on diets A to E, inclusive. The gain per gram of protein equivalent was calculated using the total

nitrogen per 100 gm of diet minus the inactive isomers times the factor 6.25 as the protein equivalent. The protein efficiency increased from 2.2 gm per gram of protein equivalent, for diet A, to 4.7 gm for diet E, containing 8% casein. The increase in protein efficiency is associated with increasing amounts of non-essential nitrogen provided by casein and by any unknown growth factors contained in it, and with a decrease in the content of inactive D-isomers.

For diet F the average daily food consumption was slightly less than that recorded for diet A, for which the food intake was the lowest of the series A to E; it was markedly less, 4.6 gm, for diet G. The protein efficiency for diets F and G, containing the larger quantities of crystalline essential acids, was somewhat higher, however, than that observed for diet A, which provided the suggested minimum quantities of these acids.

The question arises, of course, as to whether these lower food intakes were due to decreased palatability because of the larger quantities of crystalline acids. For diet F, 10.0 gm and for diet G, 13.9 gm of crystalline amino acids were present in 100 gm of diet. The larger of these quantities is considerably less than the 22.4 gm of the 10 essential and 9 non-essential crystalline acids per 100 gm of diet calculated for one of the diets fed by Rose and Fierke ('42), in which the average daily food consumption was 6.0 gm and the average daily gain 2.0 gm. Although lower palatability of diets F and G may have accounted in part for the lower food consumption, the larger quantities of crystalline acids used by Rose and Fierke did not reduce growth and food consumption below the levels obtained with these diets. The presence of the non-essential acids in the diet of Rose and Fierke suggests that they may be necessary for growth in conjunction with the essential acids. Furthermore, as has been pointed out previously, Womack and Rose ('46) more recently reported a gain of 3.4 gm per day when a mixture of 19 essential and non-essential acids were in the diet, although they did not state the quantities used.

SUMMARY

1. When the crystalline essential amino acids (supplied at the minimum levels suggested by Rose, '37) were the sole source of nitrogen, subnormal growth was obtained in the white rat.

2. Supplementation of 2, 4, 6 and 8% levels of casein with crystalline essential amino acids to meet the minimum suggested levels resulted in a progressive increase in growth rate, the highest rate, 3.9 gm per day, being noted at the 8% level of casein.

3. An increase in the quantity of essential nitrogen (supplied by the crystalline essential amino acids as the sole source of nitrogen) so that it was equal to the essential and nonessential nitrogen supplied by the 8% casein diet supplemented with crystalline acids, failed to cause an increase in growth rate significantly greater than that observed when the crystalline acids at their minimum levels were used.

ACKNOWLEDGMENTS

We take this opportunity to thank Merck and Company, Rahway, New Jersey, for the supply of crystalline amino acids, and Dr. J. L. Stokes of Merck and Company for the analyzed casein.

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# STUDIES OF THE ALLEGED GROWTH PROMOTING PROPERTY OF VACCENIC ACID [1]

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Boer, Jansen and Kentie ('46, '47a) presented evidence in support of the view that the substance responsible for the superior growth promoting activity of summer butter is vaccenic acid, an 18 carbon chain fatty acid having a double bond between the 11th and 12th carbon atoms. The isolation and proof of structure of vaccenic acid were reported by Bertram ('28), who used margarine fat and butterfat as the source materials. The configuration was found to be trans, like that of elaidic acid, and therefore Bertram referred to it as isoelaidic acid.

The reasoning which led Boer and his associates to test the growth promoting activity of this substance was unqiue. Earlier studies by Schantz et al. ('40b) as well as by Boer and others indicated that the growth promoting activity of butter-

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Some of the preliminary work involved in this research was carried out in cooperation with Dr. R. P. Geyer, now at Harvard University.

The work with synthetic vaccenic acid was made possible by the generosity of Dr. F. M. Strong and associates, who successfully synthesized both the cis and trans forms.

fat was present in certain fatty acid fractions. Distillation of the methyl esters of the butter fatty acids showed the activity to be present in the fraction containing 18 carbon atoms. The findings by Schantz et al. ('40a) that the fatty acid or acids which were responsible for the superior nutritive value of butterfat were present in the saturated fraction, and the observations of Boutwell et al. ('41) that hydrogenation of the saturated fraction decreased its nutritive value slightly, suggested that the effective agent might be an unsaturated acid whose lead soap was insoluble in alcohol, and that hydrogenation converted it into a saturated inactive compound. The fact that Boutwell et al. ('41) demonstrated improvement of the nutritional value of the unsaturated portion of butterfat following its hydrogenation can also be interpreted to mean that the same unsaturated compound was formed during hydrogenation of the original higher-unsaturated fatty acids.

All the above observations led Boer et al. ('46, '47a, '47b) to test the growth promoting activity of the only naturally occurring 18 carbon atom unsaturated fatty acid which forms a lead soap insoluble in alcohol.

The experimental evidence for the claim on behalf of vaccenic acid by Boer et al. lies in the finding that rats fed a ration containing butterfat grew better than those fed diets containing rapeseed oil, and that the addition of vaccenic acid to the latter diet at a level of 0.1% of the total ration caused the rate of growth to approach that of the animals fed butterfat. It was therefore concluded that this acid was one of the compounds in butterfat responsible for its superior nutritive value as compared with vegetable oils.

Work was started in this laboratory to determine the effect of supplementing our purified rations with vaccenic acid isolated from natural materials. The preliminary results were reported in the January 1948 issue of the Wisconsin Agricultural Experiment Station Bulletin (Geyer et al., '48).

More recently, Deuel et al. ('48) have reported on the alleged growth promoting activity of vaccenic acid. They observed no stimulatory effect on growth of young rats on either

rapeseed or cottonseed oil diets after administration of vaccenic acid or hydrogenated china wood oil. China wood oil has been reported by Boer et al. to be a good source of vaccenic acid.

Our experiments were carried out in order to study the problem further. We wished to determine whether vaccenic acid augments the growth of young rats when added to a purified diet containing corn oil, which under certain conditions has been shown to be inferior to butterfat. In this work we used vaccenic acid isolated from natural sources as well as that prepared by synthesis.

## EXPERIMENTAL

The experiments were carried out with male weanling rats of the Sprague-Dawley strain, three weeks of age and with starting weights ranging between 40–45 gm. Unless otherwise stated, each group consisted of 6 rats weighed once weekly, and growth records were of 5 to 6 weeks' duration. The rats were housed in individual metal cages with raised screen bottoms and watered and fed daily ad libitum on diets the composition of which is given in table 1.

The butterfat used in these experiments was prepared by melting, decanting and filtering fresh sweet butter obtained from the University dairy. The corn oil used was a commercial product [2] of highest grade. Special precautions were taken with all fats to prevent rancidity and off flavors. All rations were mixed weekly and kept under refrigeration.

Two levels of water soluble vitamins were used in some of the experiments because it was previously reported that the difference in growth rates of rats fed butterfat or corn oil depends on the level of the B vitamins in the diet (Boutwell et al., '45).

Vaccenic acid was prepared according to the method of Bertram ('28). The same procedure was also employed by the Dutch workers. The final product we obtained was very simi-

[2] Mazola.

lar on the basis of iodine number and melting point to that of Bertram and Boer et al. We used these properties as the criteria for the purity of the material isolated from natural products. We believed that in our first 6 experiments the preparation used was not pure vaccenic acid, because it had

TABLE 1

*Composition of the diets*

| BASAL MIXTURE | | | | | VITAMIN SUPPLEMENTS PER 100 GM OF BASAL MIXTURE | | |
|---|---|---|---|---|---|---|---|
| Components | Diet | | | | | Medium level | High level |
| | 1 | 2 | 3 | 4 | | | |
| | % | % | % | % | | mg | mg |
| Fat [1] | 28 | 28 | 10 [2] | 28 | Thiamine | 0.20 | 0.50 |
| Casein [3] | 20 | 20 | 5 | .. | Riboflavin | 0.30 | 0.70 |
| Salts [4] | 4 | 4 | 3 | .. | Pyridoxine | 0.30 | 0.60 |
| Sucrose | 48 | .. | .. | .. | Ca pantothenate | 1.50 | 3.0 |
| Lactose | .. | 48 | .. | .. | Choline | 150.00 | 150.00 |
| Rice (polished) | .. | .. | 72 | .. | Folic acid | ... | 0.20 |
| Yeast (dried) | .. | .. | 10 | .. | Biotin | ... | 0.02 |
| Skim milk powder [5] (mineralized) [6] | .. | .. | .. | 72 | Niacin | ... | 0.60 |
| | | | | | *p*-Aminobenzoic acid | ... | 15.00 |
| | | | | | Inositol | ... | 50.00 |
| | | | | | Carotene | 0.56 | 0.56 |
| | | | | | Tocopherol | 2.24 | 2.24 |
| | | | | | Calciferol | 0.014 | 0.014 |
| | | | | | 2-Me-1,4-napthaquinone | 0.21 | 0.21 |

[1] Butterfat or corn oil or corn oil containing 1% of vaccenic acid.

[2] Olive oil replaced corn oil in this experiment.

[3] Extracted for three two-hour periods with boiling 95% alcohol.

[4] Phillips and Hart ('36).

[5] Extracted for 4 8-hour periods with diethyl ether.

[6] Ten grams of the completed ration contain 0.6 mg each of copper sulfate and manganese sulfate and 12 mg of iron pyrophosphate.

an iodine number of 81.2 and melting point of 34°C. (Wiley method). Bertram ('28) reported I.N. 88 and melting point 38°C. We also observed that we could get purer material by increasing the number of recrystallizations from acetone at low temperatures. Butterfat was not a good source material because it contained only 0.8–0.9% of vaccenic acid, and therefore we looked for a richer source. A commercially available

hydrogenated fat,[3] was found to be a good source of vaccenic acid (yield about 3%) and from this we could isolate larger amounts of purer material. The final preparation made from this source had an iodine number of 87.6 and melting point of 38°C. In certain experiments, the results of which are reported in table 3, this preparation was used.

Recently Ahmad, Bumpus and Strong ('48) synthesized both the cis and the trans forms of vaccenic acid. The melting point of the cis form of the acid was 5°C. and that of the trans isomer was 42–43°C. The iodine number and other physical constants of the compound as well as its chemical characterization by these workers, indicated the identity of its properties with those demanded theoretically for vaccenic acid.

The high melting point of the trans form of vaccenic acid has also been reported by Rao and Daubert ('48), and it is therefore obvious that the material isolated from natural sources by any other group of workers was in no case absolutely pure vaccenic acid.

The results of three experiments on the supplementary effects of vaccenic acid are given in table 2. It will be noted from these results that the differences in growth resulting from the butterfat and corn oil diets, even on medium levels of vitamins, were not significant. Furthermore, supplementation with vaccenic acid did not increase the growth of rats on the corn oil diets. Of the 6 groups of animals fed supplements of vaccenic acid, only one showed a slight increase in growth, which, however, was within the experimental variation generally observed in biological studies of this type. In other cases the acid appeared to have a slightly depressing effect on the growth of the animals. No other explanation, except possibly biological variation, could be held responsible for this effect because chemical examination did not indicate the presence of lead, chlorine or other contaminating material in the vaccenic acid preparations.

On the basis of previous reports from this laboratory (Boutwell et al., '43) that the difference between butterfat and

[3] Crisco.

corn oil is considerably greater when lactose is the carbohydrate in the diet, it was decided to repeat the experiments with this carbohydrate replacing sucrose. Two such experiments with lactose (diet no. 2) were carried out. In both the superiority of butterfat over corn oil on a lactose diet was again demonstrated. On the other hand, the vaccenic acid was found to have no effect as a supplement to corn oil diets even under these conditions.

TABLE 2

*Effects of additions of vaccenic acid to corn oil diets*
*(Figures represent the average number of grams gained, range is shown by numbers in parentheses. Six rats in each group)*

| CARBOHYDRATE USED | DIET NO. | EXPT. NO. | NO. OF WEEKS | VITAMIN LEVEL | BUTTERFAT | CORN OIL | CORN OIL + VACCENIC ACID |
|---|---|---|---|---|---|---|---|
| Sucrose | 1 | 1 | 6 | Medium | 173 (162–187) | 175 (149–216) | 174 (171–186) |
| | | | | High | 197 (178–220) | 186 (162–201) | 190 (165–225) |
| Sucrose | 1 | 2 | 5 | Medium | 160 (145–188) | 153 (147–163) | 150 (126–192) |
| | | | | High | 174 (160–188) | 156 (149–164) | 150 (140–160) |
| Sucrose | 1 | 3 | 5 | Medium | 159 (129–201) | 153 (130–186) | 141 (127–149) |
| | | | | High | 170 (141–191) | 187 (176–198) | 178 (131–212) |
| Lactose | 2 | 4 | 6 | Medium | 130 (115–145) | 122 (103–134) | 122 (112–143) |
| | | | | High | 147 (138–158) | 143 (128–158) | 138 (128–150) |
| Lactose | 2 | 5 | 6 | Medium | 161 (127–200) | 128 (114–154) | 126 (116–145) |
| | | | | High | 154 (140–189) | 141 (123–152) | 129 (105–152) |
| Polished rice | 3 | 6 | 5 | .... | 166 (160–176) | 163 [1] (150–174) | 154 [2] (127–185) |

[1] Olive oil.
[2] Olive oil containing 1% vaccenic acid.

After failing to observe on our purified diets any growth promoting effects of vaccenic acid, it was decided to determine whether this activity of the material could be obtained with a natural diet, as closely resembling that used by Boer et al. ('46, '47a, '47b) as possible. The diet used by them consisted of 72% polished rice, 5% extracted casein, 10% dried yeast, 3% salt mixture and 10% fat (butterfat or rapeseed oil or rapeseed oil supplemented with vaccenic acid). The diet we used differed from theirs only in the replacement of the rapeseed oil with olive oil. We made our initial trials with rapeseed oil obtained in the local market but due to its poor quality, mostly due to rancidity, we decided not to use it.

Again 6 male rats were placed in each group; their average 6-week weight gain in grams is indicated in table 2, experiment 6. Here again no stimulatory effect of vaccenic acid could be detected. The growth of the animals on butterfat and olive oil diets was also found to be nearly equal. It should be emphasized that the starch of polished rice was the main carbohydrate in the diet, and this confirms the report of Boutwell et al. ('43) that when starch is the dietary carbohydrate there is no difference between the growth promoting activity of butterfat and that of the vegetable fats studied.

As mentioned before, all the above experiments were carried out with weanling three-week old male rats obtained from the Sprague-Dawley Company. On the assumption that weanlings might carry from their mothers a store of vaccenic acid and therefore obliterate the possible beneficial effect of feeding this acid, we thought it desirable to test the activity of vaccenic acid by using young rats depleted of this material. With this in mind, we depleted young female rats (future mothers of the experimental weanlings) by keeping them on diet no. 1 with corn oil as the dietary fat. This was done in view of our previous finding that body fat obtained from rats fed a purified diet containing corn oil as the only dietary fat contained no vaccenic acid (Geyer et al., '47b). For purposes of comparison we also used weanlings from mothers on diet no. 1 with butterfat as the source of fat. In addition, two groups of

females were placed on a skim milk powder diet (table 1, diet no. 4), one group receiving butterfat, the second corn oil.

When the females matured they were mated with normal males. Their young were selected in groups of three weanling litter mates of the same sex and weight and placed on three diets: one on butterfat, the second on corn oil, and the third on corn oil containing 1% vaccenic acid. The young from the sucrose-fed mothers were placed on purified sucrose diets, while those from the skim milk mothers were placed on skim milk diets. Table 3 gives the results.

TABLE 3

*Effects of vaccenic acid supplementation on corn oil diets of depleted young (Figures represent the average number of grams gained in 6 weeks; range is shown by numbers in parentheses)*

| SERIES | DIET OF THE MOTHER | SEX | NO. OF TRIOS OF LITTER MATES | BUTTERFAT | CORN OIL | CORN OIL + VACCENIC ACID |
|---|---|---|---|---|---|---|
| I | Butterfat mineralized skim milk | M | 6 | 126 (104–151) | 110 (97–119) | 105 (91–118) |
| II | Corn oil mineralized skim milk | M | 9 | 148 (122–165) | 126 (110–148) | 126 (109–151) |
| III | Butterfat-sucrose | M | 7 | 136 (120–159) | 122 (102–140) | 131 (103–152) |
| IV | Corn oil-sucrose | M | 9 | 125 (105–142) | 121 (108–138) | 116 (98–141) |

It was again observed that vaccenic acid produced practically no beneficial effect on the rate of growth of young rats. Only in series 3, where the young were obtained from butterfat-sucrose-fed mothers, was the average growth of the group supplemented with vaccenic acid higher than that of the unsupplemented group. But even here a stimulatory effect was observed in only 4 out of the 7 groups of animals employed in the series, while in the other three groups no effect was noticeable. Considering the individual variations often

observed among animals in an experiment of this type, it is doubtful whether these higher values are significant.

In view of the fact that we always had some doubt about the purity of the preparation isolated from natural sources, we decided to reinvestigate the problem by using synthetic vaccenic acid, prepared in this laboratory by Ahmed, Bumpus and Strong ('48). In our first trial we used the cis form of the synthetic compound at a level of 1% of the total fat administered. The plan of the experiment was identical with

TABLE 4

*Effects of additions of synthetic vaccenic acid on the growth of young rats (Six animals in each group)*

| FAT | FORM OF VACCENIC ACID | LEVEL OF VACCENIC ACID | AVERAGE GAIN IN WT. IN 5 WKS. | |
|---|---|---|---|---|
| | | | Expt. I | Expt. II |
| | | % in fat | gm | gm |
| Butterfat | ... | .. | 107 | 107 |
| Corn oil | ... | .. | 90 | 88 |
| Corn oil supplemented with vaccenic acid | cis | 1.0 | 87 | 80 |
| Corn oil supplemented with vaccenic acid | cis | 0.3 | | 75 |
| Corn oil supplemented with vaccenic acid | cis | 0.1 | | 86 |
| Corn oil supplemented with vaccenic acid | trans | 1.0 | | 85 |

that described earlier in connection with experiments 4 and 5 (table 2). Lactose was used as the sole carbohydrate of the diet, and the medium level of the B vitamins was supplied (table 1). The results are given in table 4.

Although over a period of 5 weeks the weight gains of the animals on the supplemented or unsupplemented diets were exactly equal for some unexplained reason, in the second and third week the average growth of the group receiving vaccenic acid was somewhat better than that of their controls. In order to check the possibility that the amount of vaccenic acid which we were administering to the animals was too high and was

possibly producing some undesirable effect, we decided to lower the level of vaccenic acid in the diet to ⅓ and 1/10 of the amount we gave before. It should be mentioned that Boer and his group ('46, '47a, '47b) used 0.1% of vaccenic acid in their diets, but in practically all of our experiments we used the vaccenic acid at a level of 0.28% of the total diet.

At this time the trans form of the synthetic vaccenic acid was also made available and its activity tested. Table 4 gives the results with both the cis and trans forms at different levels of intake. No stimulatory effect of either form of vaccenic acid at any level could be observed.

## DISCUSSION

Under our experimental conditions the work described demonstrated conclusively that the rate of growth of rats is little affected whether the diet contains vaccenic acid or not. This was found to be true whether the basal diet contained as the main carbohydrate sucrose, lactose or starch, or the dietary fat used was corn oil or olive oil, or whether the levels of the water soluble vitamins were medium or high. Neither were there any differences when vaccenic acid was isolated from natural fats or made by synthesis. The results also confirm the report of Boutwell et al. ('43) that butterfat is always superior to corn oil when lactose is the sole carbohydrate of the diet.

Recent studies by Jack and Hinshaw ('47) on the nutritive value of fractions obtained by cold crystallization of milk fat also indicate that some other factor or factors must be responsible for the superior growth promoting activity of milk fat. These authors reported the —53°C. filtrate fraction to have a growth promoting action much superior to that of the milk fat itself. In view of the high melting point of vaccenic acid it would appear that after crystallization at —20°C. all of this compound would be left in the precipitate, yet the growth gain and efficiency of utilization of the diet on this fraction (—20°C.) as the source of dietary fat were the least of the group when the diet was compared with those contain-

ing the original milk fat and the other fractions. The existence of such a fraction, having growth stimulating properties superior to the original butterfat, has also been observed in our recent studies on the nutritive value of a fraction of butterfat prepared by cold crystillization (Geyer et al., '47a; Nath et al., '48).

The negative effect of vaccenic acid on the growth of rats also became apparent in our recent studies on the comparative nutritive values of commercial hydrogenated cottonseed oil [4] and corn oil (unpublished data). In spite of the fact that we can isolate from the hydrogenated product about 3% of material presumably corresponding to vaccenic acid, this fat did not support better growth of young rats than did corn oil.

From all these experiments it is obvious that vaccenic acid has no special growth stimulating properties. The fact still remains that there is something present in butterfat at certain seasons of the year which may be either a definite chemical compound or, more likely, a fraction rich in certain favorable fatty acids. This factor or factors concentrated by cold fractionation from acetone has greater growth stimulating properties than corn oil or the butterfat itself.

## SUMMARY

1. No increase in the growth of young rats on diets containing either corn oil or olive oil resulted from supplementing the diets with vaccenic acid isolated from either butterfat or commercial hydrogenated cottonseed oil.

2. Replacing the sucrose by lactose or starch (rice) as the source of carbohydrate in the diets, or changing the levels of the water soluble vitamins, did not alter the results as far as the vaccenic acid supplementation was concerned.

3. Similar results were obtained when weanling rats from depleted mothers were used.

4. Supplementing a corn oil-lactose diet with either the cis or trans forms of synthetic vaccenic acid produced no growth stimulating effects.

[4] See footnote 3, page 765.

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# CALORIC RESTRICTION AND PROTEIN METABOLISM IN THE GROWING MOUSE [1]

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SEVEN FIGURES

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It is a well-established principle of nutrition that restrictions in caloric intake below certain minimum requirements result in a decreased retention of nitrogen. For example, Deuel ('48) cites evidence that in man a maximum protein-sparing effect of non-protein calories may be noted with intakes of 1500 calories per day but not when the caloric level is reduced to 600 calories per day. In the growing animal it is possible to adjust caloric intake and to measure nitrogen retention with great accuracy. Employing rats and mice, Bosshardt et al. ('46a) have presented evidence that with increasing restriction of calories protein utilization remains constant until a critical caloric intake is reached. Restriction beyond this point results in a very rapid diminution in the efficiency of protein retention. Benditt et al. ('48) have shown that this same phenomenon is apparent in the protein-deficient rat during a period of repletion. In fact, the critical caloric level for optimum protein utilization in the rat was essentially identical in both of these studies.

Deuel ('48) has reviewed a number of investigations which show that the protein-sparing effect of carbohydrate is considerably greater than that of fat when subjects are ingest-

[1] A preliminary report of this data was presented before the American Institute of Nutrition, Chicago, May 22, 1947.

ing the same quantities of protein. Furthermore, in complete protein starvation carbohydrate, but not fat, has the property of sparing body protein. Conclusions reached by various investigators (Landergren, '03; Best and Taylor, '39) make it appear logical that protein-sparing can be related to three different metabolic pathways: (1) Protein may act as a precursor of glucose and thus be diverted into the formation of essential carbohydrate in complete or severe dietary carbohydrate deprivation; (2) intermediary products of carbohydrate metabolism may be available for the resynthesis of amino acids by utilizing some of the nitrogen released by the catabolism of protein; (3) both dietary fat and carbohydrate supply essential calories and in their absence protein must be diverted to the production of calories.

In considering the above factors that may regulate protein-sparing, it would seem likely that equal caloric intakes of fat or carbohydrate would spare protein to the same extent if some quantity of carbohydrate were present in the diet. Allison, Anderson, and Seeley ('46) maintained dogs on a markedly restricted caloric intake and found, as was to be expected, that the nitrogen balance index decreased; fat or carbohydrate added to the calorically deficient intake increased the nitrogen balance index to the same extent.

The studies presented here were designed to investigate the growth promoting effects of fat, carbohydrate and protein. All were conducted on the growing mouse receiving caloric intakes that were known to be below the minimum level for optimum protein utilization.

## EXPERIMENTAL METHOD

Food utilization was studied with growing mice throughout a wide range of caloric intakes. Two types of feeding were employed, ad libitum and restricted. Two protein sources, casein [2] and wheat gluten, were used. Although there is an appreciable difference in their nutritive qualities, both sources

[2] Borden's Labco.

are able to support good growth in the mouse if sufficient protein is fed.

The basal diet in the ad libitum experiments consisted of 2% corn oil [3], 20% glucose [4], 4% salt mixture (Hubbell, Mendel and Wakeman, '37), 2% cellulose [5], and 1% Wilson's 1:20 liver concentrate powder, and was supplemented so that each 100 gm of diet contained 4 mg of α-tocopherol, 900 U.S.P. units of vitamin A, 180 U.S.P. units of vitamin D, 1 mg of 2-methyl-1,4-naphthoquinone diacetate, 0.8 mg of thiamine hydrochloride, 1.6 mg of riboflavin, 0.8 mg of pyridoxine hydrochloride, 4.0 mg of niacin, 4.4 mg of calcium pantothenate, 4.0 mg of para-aminobenzoic acid, 200 mg of choline chloride, and 21.6 mg of inositol. The remainder consisted of the protein source, hydrogenated cottonseed oil [6] and white dextrin, the levels being varied to obtain diets with different protein levels and caloric densities.

Diets with varying protein levels and caloric densities, using casein and wheat gluten as the protein sources, were fed ad libitum to groups of 7 male albino weanling mice (Sharp and Dohme, Swiss Webster strain) for 10-day periods. Records of the body weight gains and of the food and protein intakes of the test animals were kept. Only the data obtained from those groups in which the average protein intake corresponded to that giving maximum utilization with isocaloric diets were considered in this report. Intakes giving maximum utilization were 0.19 gm of casein per mouse per day and 0.60 gm of wheat gluten per mouse per day (Bosshardt and co-workers, '46b).

In the restricted feeding experiments the daily protein intakes per mouse were maintained at 0.19 gm of casein and 0.60 gm of wheat gluten. The daily intakes of the vitamins, minerals, and roughage were also constant and were the amounts present in 2 gm of the basal diet. An intake of 2 gm per mouse per day is the average intake of the basal diet

[3] Mazola.
[4] Cerelose.
[5] Cellu flour.
[6] Primex.

containing 10% casein and 25% fat under conditions of ad libitum feeding for a 10-day period. Differences in caloric intake were obtained by varying the daily intakes of fat and white dextrin.

For the calculation of caloric intakes the constants employed were 9.3 cal. per gram of fat, 4.0 cal. per gram of wheat gluten, 4.4 cal. per gram of casein, 3.75 cal. per gram of glucose, and 4.23 cal. per gram of dextrin. Caloric intakes were calculated on the basis of the average body weight $^{2/3}$. This was as-

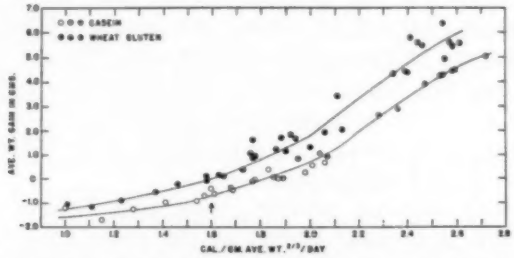


Fig. 1 The relationship between body weight gain and caloric intake with the two protein sources, casein and wheat gluten. The graphic symbols used are ⓘ and ◑ for ad libitum feeding, ○ and ● for increases in caloric intake by increasing intake of carbohydrate from ↑, and ⊖ and ◒ for increases in caloric intake by increasing intake of fat from ↑.

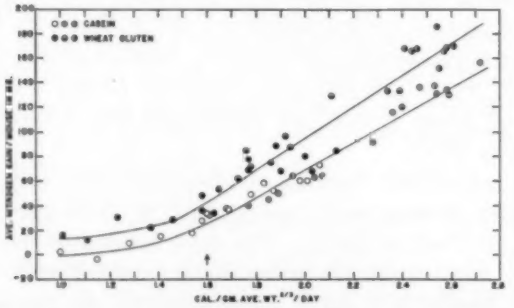


Fig. 2 The relationship between body nitrogen gain and caloric intake with the two protein sources, casein and wheat gluten. The graphic symbols used are ⓘ and ◑ for ad libitum feeding, ○ and ● for increases in caloric intake by increasing intake of carbohydrate from ↑, and ⊖ and ◒ for increases in caloric intake by increasing intake of fat from ↑.

sumed to be proportional to average body surface area during the 10-day period.

## RESULTS

When the average weight gains or body nitrogen gains were plotted against the caloric intakes per unit of body surface area, it was found that at any given caloric intake the animals receiving the wheat gluten grew better than did those receiving casein (figs. 1 and 2). Each point in the figures represents the average of 7 mice. A similar relationship was found when the caloric intake per unit of body surface area was plotted against the "caloric efficiency ratio" (gm gain in weight per cal. consumed) or against the body calorie equivalent based on the fat and protein contents (figs. 3 and 4).

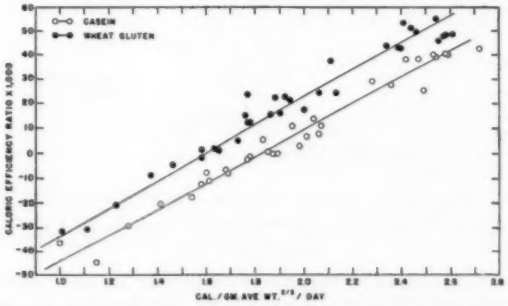


Fig. 3 Relationship between caloric intake per unit body surface area and the "caloric efficiency ratio" with mice fed casein and wheat gluten.

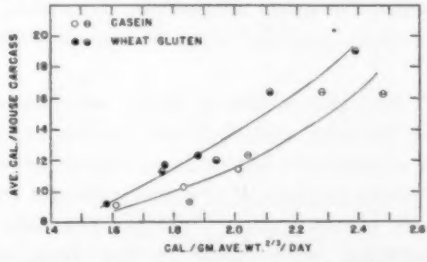


Fig. 4 Relationship between caloric intake per unit body surface area and the body calorie content based upon the fat and protein contents. ○ and ● indicate caloric intake increases by additional carbohydrate and ⊖ and ◒ by additional fat.

The protein (N × 6.25), fat, and water contents accounted for 94 to 96% of the total body weight. In no case was there a difference between the effects of fat or carbohydrate, the response per calorie being the same by all methods of calculation.

As the caloric intake was decreased, the difference in the nutritional quality of the two proteins was minimized (fig. 5). However, only a very small fraction of the ingested protein was used for tissue synthesis when the caloric restriction was severe. It would appear that throughout the range of caloric

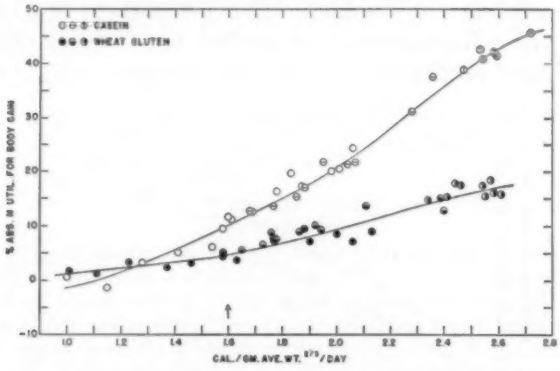


Fig. 5 The relationship between the caloric intake per unit body surface area and the growth utilization of dietary protein expressed as the percentage of absorbed nitrogen utilized for body nitrogen gain.

intakes studied differences in growth were due more to the quantity of protein supplied than to the nutritive quality of that protein.

In order to test this point, a study was made in which a portion of the fat and carhohydrate in the casein diets was replaced with equicaloric amounts of casein or wheat gluten, so that the protein intakes of the test animals were the same as in the wheat gluten series (0.60 gm per mouse per day). These replacements were made in the diets supplying 5.5 to 7.0 cal. per mouse per day. This range is approximately 50 to 65% of the caloric intake when feeding is ad libitum.

This replacement of non-protein calories resulted in an increase in growth and body nitrogen gain to that previously obtained with the wheat gluten feeding (figs. 6 and 7). The effects were the same when the additional protein was either casein or wheat gluten.

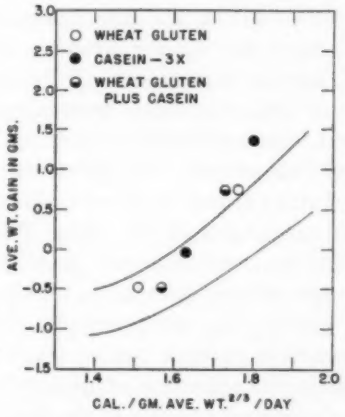


Fig. 6 The effect of replacing non-protein calories with protein on the weight gains of mice subjected to a caloric restriction. The two lines are portions of the curves shown in figure 1.

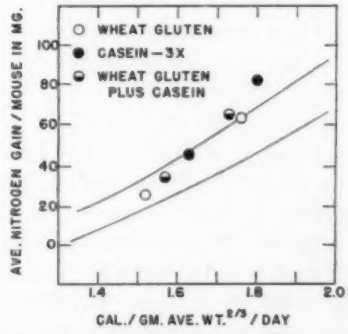


Fig. 7 The effect on the body nitrogen gains of mice subjected to a caloric restriction of replacing non-protein calories with protein. The two lines are portions of the curves shown in figure 2.

## DISCUSSION

The major factor limiting growth throughout the wide range of caloric intakes studied in the present investigation was the amount of protein ingested. At the level of adequate calorie consumption amounts of wheat gluten and casein were fed that would give optimum efficiency of protein utilization for growth. These levels of intake were not adequate for an optimum growth rate for either of the proteins studied. If the intake of casein was increased three-fold so as to equal the intake of wheat gluten, percentage utilization of the casein dropped from approximately 46 to 24 (unpublished data). This means that protein utilization was roughly halved by increasing the intake three times and, therefore, additional protein deposition must have taken place. When a restriction in caloric consumption was imposed so that approximately half of an adequate caloric intake was being supplied (1.6 cal./gm ave. wt.$^{.75}$/day), the same general relationship held. A three-fold increase in protein intake (casein) caused a decrease by one-half in the efficiency of utilization. Again extra protein deposition resulted, in spite of the fact that in this case there was a concurrent deficiency in calories.

As the severity of caloric restriction was increased the decrease in efficiency of utilization of the protein from the two sources appeared to proceed at different rates, so that at very low caloric intakes the percentage of absorbed nitrogen utilized for body gain became the same for both proteins. This method of expressing protein utilization is complicated by the fact that wheat gluten was consumed in an amount three times greater than that of casein. On the basis of body nitrogen stored, the two protein sources maintained approximately the same relationship to one another throughout the entire range of caloric intakes. When caloric restriction was severe, the percentage utilization of protein became so low that minor differences in the nutritive values of different proteins were no longer of major importance and the over-all effect of protein nutrition was governed primarily by the quantity of protein ingested.

The conclusion seems justified that in conditions of calorie undernutrition the efficiency of protein utilization is governed largely by the extent of the caloric restriction, and that within certain limits increased amounts of protein can be utilized by the body if the protein intake is increased, even though no change in calorie consumption is effected. If caloric consumption is increased, protein will be utilized by the body to a greater extent, even though the protein intake is held constant. However, if calories are increased by adding protein rather than non-protein nutrients, the amount of protein utilized will be still larger. This means that in many states of semi-starvation protein is the most important limiting factor.

From the data given in figure 4 it was possible to calculate the approximate changes in energy expenditure that accompanied restriction in caloric intake. It was estimated that with a 34% decrease in energy intake (2.39 to 1.53 cal./gm ave. wt.$^{3/4}$/day) there was a 14% decrease in energy expenditure (21.6 to 15.7 cal./gm ave. wt.$^{3/4}$/day). This shows that in the growing animal on a constant protein intake energy conservation is accomplished by a reduction in basal metabolism. This, of course, has been observed in semi-starvation by many investigators.

Another point of interest is the observation that fat and carbohydrate are equal in their protein-sparing effect. It must be kept in mind that some carbohydrate was present in all diets, so this observation in no way contradicts earlier studies showing a complete lack of protein-sparing by fat under conditions of total carbohydrate deprivation. In addition, relatively large amounts of protein were present in all diets. As the caloric restrictions became more severe, increasing amounts of protein were catabolized and thus were undoubtedly available for conversion into carbohydrate.

The results obtained in this study elaborate and confirm the results of Elman, Davey, and Kiyasu ('45), who found that positive nitrogen balance could be maintained on a low

caloric intake if sufficient protein were fed, but not when a portion of the protein was replaced by carbohydrate.

## SUMMARY

The effects of restricting the caloric intake by decreasing the consumption of fat and carbohydrate while holding constant the protein, vitamin, and mineral intakes have been studied in growing mice. Under such conditions there were observed: (1) a decrease in growth rate; (2) a decrease in the efficiency of protein and calorie utilization for growth; and (3) a decrease in energy expenditure.

Extra quantities of dietary protein caused increased growth to approximately the same extent in mice receiving an adequate caloric intake or a caloric intake restricted to about one-half the adequate level.

With low caloric intakes, extra calories in the form of protein caused a much greater growth response than equivalent calories supplied as fat or carbohydrate.

Under the conditions of this study, in which all diets contained some fat, carbohydrate, and protein, fat and carbohydrate were equal in their protein-sparing effect.

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# THE EFFECTS OF ORAL ADMINISTRATION OF DIFFERENT PROTEINS ON THE PLASMA PROTEINS OF PROTEIN-DEPLETED DOGS

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## INTRODUCTION

The nutritive quality of a protein can be measured biologically in a variety of ways, among which the growth method of Osborne and Mendel ('26), the biological value method of Mitchell ('44), and the nitrogen balance index method of Allison and Anderson ('45) are the most commonly used. Madden and Whipple ('40) believe that the depletion of plasma proteins puts a strain upon the body and accentuates the normal demand for plasma protein production, perhaps even at the expense of building other tissue proteins. This hypothesis was utilized by them and by other investigators (Cox and Mueller, '44; Holman et al., '34; Melnick et al., '36a; Seeley, '45; and Weech and Goettsch, '38a) as a basis for the measurement of the nutritive values of proteins in terms of their ability to stimulate the production of plasma proteins in standardized dogs depleted of proteins. The results of their studies reveal that not only do various proteins differ in their ability to regenerate plasma protein, but some favor the production in the plasma of more globulins than albumin. The latter view was substantiated by the re-

sults (Chow et al., '48) of electrophoretic analysis of the plasma of protein-depleted dogs made before and after protein hydrolysate feeding. Such a study permits a more complete analysis of the globulin fractions and a more precise determination of the albumin. The results indicate that lactalbumin hydrolysate feeding stimulates the regeneration of albumin but not of globulins, whereas casein hydrolysate promotes the production of both albumin and globulins.

The above study was extended to include the oral feeding of 5 whole proteins commonly present in our daily diets, in order to ascertain whether whole proteins, like the hydrolysates, also differ both qualitatively and quantitatively in their ability to regenerate different components of plasma proteins. Tryptic digests of casein and lactalbumin, whose chemical and biological properties were described by Bolling et al. ('47), were included in order to ascertain whether a hydrolysate which presents to the body for tissue synthesis a whole pattern of amino acids and peptides, available at one time, might not be superior to the whole proteins, which must be digested and then synthesized. Lastly, it is of interest to ascertain whether the same proteins are qualitatively similar when different methods of measurement are used. The results of our investigation are presented in this paper.

## EXPERIMENTAL

### *Depletion of the protein reserve in dogs*

Normal animals can be depleted of their protein reserves by plasmapheresis, by prolonged protein-free feeding, or by feeding a diet with inadequate caloric intake but an otherwise sufficient amount of nutritionally adequate protein to maintain nitrogen balance. In the last situation (Lusk, '28; Elman, '44), protein is used primarily to supply the necessary energy. Any one of the above procedures, or a combination of them, should provide us with a means of protein depletion. Therefore, attempts were made to so deplete three groups of healthy normal dogs of their protein reserves, in order to

ascertain whether protein depletion, regardless of its cause, will result in similar changes in the electrophoretic patterns of the plasma proteins, and in the total circulating plasma proteins (plasma volume × plasma protein concentration). The procedures used for the estimation of plasma volume, total plasma protein concentration, and electrophoretic analyses have already been described by Chow, Seeley, Allison, and Cole ('48).

The first group of dogs (Group A), totaling 15 animals, was kept on a protein-free diet and plasmapheresed once a week by withdrawing approximately one-fourth of the total blood volume calculated on the basis of their body weight. The blood was citrated and centrifuged and the plasma was removed. The red cells were washed once with a sterile 0.85% sodium chloride solution and suspended in a normal saline solution equal in volume to that of the plasma removed (Seeley, '45). The suspension was returned to the animals intravenously. Plasmapheresis was repeated as often as necessary in order to bring the plasma protein concentration to about 4.5 to 5.2 gm %. Its frequency was dependent on the adequacy of the initial protein reserve.

The second group of 15 normal animals was put on a protein-free diet calculated to supply 80 cal. per kg body weight per day. The composition of the diet was that used by Allison and Anderson ('45). In our experience, this procedure of depletion required from 6 to 8 weeks to lower the plasma protein concentration to the desired level. A third group of 5 animals was put on a casein diet containing 0.20 gm of casein nitrogen and 30 cal. per kilogram body weight per day. This diet was prepared by replacing sucrose in the protein-free diet with casein. Two hundred milligrams of casein nitrogen were sufficient to keep normal dogs with adequate protein reserves in nitrogen balance, provided no less than 60 cal. per kg body weight per day were supplied. To deplete dogs on this partial starvation diet required three to 4 weeks.

The results (see table 1) of analyses of the composition of plasma protein before and after such depletion demon-

strate that each of the three methods of depletion brought about a decrease in plasma volume and plasma protein concentration, and therefore total circulating plasma proteins, by 35 to 40%. In terms of percentages the outstanding changes were a sharp decrease in albumin and a marked increase in the alpha globulins. In terms of the circulating plasma proteins, the albumin decreased markedly and the gamma globulin tended to decrease slightly; whereas the alpha globulins changed but little or were slightly increased.

TABLE 1

*Composition of plasma protein of dogs before and after protein depletion by different methods*

| METHOD OF DEPLETION | NO. OF DOGS USED | TREATMENT | PLASMA VOLUME | PLASMA PROTEINS | | | | TOTAL CIRCULATING PLASMA PROTEINS | | | A/G |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Total | Fractions | | | Albumin | Globulins | | |
| | | | | | Albumin | Globulins $\alpha_1 + \alpha_2$ | Globulins $\gamma$ | | $\alpha_1 + \alpha_2$ | $\gamma$ | |
| | | | *ml* | *gm%* | % | % | % | *gm* | *gm* | *gm* | |
| A [1] | 15 | Control | 473 | 6.21 | 42 | 16 | 9 | 12.4 | 4.5 | 2.5 | 0.75 |
| | 15 | Depleted | 427 | 4.48 | 21 | 26 | 9 | 4.0 | 4.9 | 1.8 | 0.27 |
| B [2] | 15 | Control | 603 | 6.74 | 40 | 16 | 8 | 16.0 | 6.6 | 3.0 | 0.67 |
| | 15 | Depleted | 510 | 5.04 | 21 | 26 | 11 | 5.3 | 6.8 | 3.0 | 0.27 |
| C [3] | 5 | Control | 500 | 6.37 | 40 | 15 | 9 | 12.7 | 4.8 | 2.9 | 0.67 |
| | 5 | Depleted | 425 | 4.52 | 21 | 25 | 10 | 3.8 | 4.5 | 1.6 | 0.27 |

[1] By plasmapheresis and protein-free feeding.
[2] By protein-free feeding alone.
[3] By feeding a diet low in calories.

Since any one of the three methods gave essentially the same results, the protein-free feeding method without plasmapheresis was chosen for further experiments because of its convenience and the minimum exposure of the animals to possible infections during plasmapheresis. In order to standardize our depleted dogs as much as possible, the protein-free feeding was continued only until the albumin to globulin ratio levelled to 0.2 to 0.3. Most animals did not survive further depletion. We believe that these dogs were as severely depleted as if they had also been plasmapheresed.

### *Chemical and nutritive properties of test proteins*

Five commonly consumed proteins of varying nutritive quality (whole egg, egg white, lactalbumin, casein, and wheat gluten) were used for the repletion studies. Enzymatic digests of casein and lactalbumin were also included for comparison with the respective undigested proteins.

TABLE 2

*The chemical and biological properties of the proteins and the protein hydrolysates used*

| SUBSTANCES | N | DEGREE OF HYDROLYSIS [1] | NITROGEN BALANCE INDEX [2] | MAXIMUM PROTEIN EFFICIENCY [3,4] | CANNON RAT DEPLETION [4,5] |
|---|---|---|---|---|---|
| | % | | | | |
| Egg white | 15.0 | 0 | 1.1 | 3.3 | 6.4 |
| Lactalbumin | 13.0 | 0 | 1.0 | 3.2 | 5.8 |
| Whole egg | 11.8 | 0 | 0.95 | 1.8 | 5.6 |
| Casein | 13.8 | 0 | 0.8 | 2.3 | 5.5 |
| Wheat gluten | 13.2 | 0 | 0.3 | 0.7 | 2.9 |
| Lactalbumin hydrolysate | 12.7 | 35 | 1.0 | 3.1 | .. |
| Casein hydrolysate | 13.5 | 25 | 0.8 | 2.2 | .. |

[1] Degree of hydrolysis = increase in amino nitrogen (the nitrous acid method) calculated as per cent of the total nitrogen.

[2] Nitrogen balance indexes were determined in normal dogs by the method of Allison and Anderson ('45).

[3] Protein efficiency was determined in mice using the method of Osborne and Mendel ('26). The mice were fed at 5 levels of nitrogen intake.

[4] The authors are indebted to Dr. A. Black for these measurements.

[5] Proteins were fed to rats depleted of protein reserves according to the procedure of Cannon ('45). Protein efficiency was measured by determining the weight gain of these animals per gram of food nitrogen consumed.

Since these test proteins were not chemical entities and their chemical composition and nutritive value might be subject to variation in commercial processing, a few of their chemical and biological properties are shown in table 2. The results demonstrate that with respect to nitrogen balance index, a measure of the amount of protein nitrogen necessary to

maintain nitrogen balance in normal dogs, and the growth efficiency, a criterion of ability to support growth in normal mice or to replace body tissues lost due to depletion (Cannon's technique, '45), these 5 proteins and the two protein hydrolysates are, as is commonly believed, in the following order of value: egg white and lactalbumin, whole egg, casein, and wheat gluten. The hydrolysates of lactalbumin and casein have the same nutritive values as their original proteins.

The liver protein regeneration properties of these proteins were also determined by us using the Harrison and Long ('45) procedure. However, we found that the caloric intake must be sufficient; otherwise the results were not reproducible. The following procedure, which yielded satisfactory results, is given in detail:

Healthy normal rats of either sex, weighing approximately 225 gm, were fed ad libitum a stock diet consisting of casein, corn, starch, and mazola with adequate vitamin and salt supplements. A week prior to the experiments these rats were weighed every other morning. Only those animals which showed constant weights ($\pm$ 3 gm) within this period were pooled. Ten rats, the control group, were sacrificed at this time. The remaining animals were given only water ad libitum for 48 hours in order to deplete their liver proteins. Ten of these animals (the fasted group) were sacrificed. The remaining animals were divided into groups of 10 each and were then fed test diets containing 40% protein for 4 successive days (10 gm of diet for the first day and 8 gm for the next three days). It was found that, as a rule, the rats consumed all the rations as scheduled; animals that did not were discarded. The composition of the diet was 40% test protein, 22% a partially hydrolyzed starch preparation,[1] 8% yeast, 4% salt, 2% codliver oil, 24% hydrogenated cottonseed oil.[2] Twenty-four hours after the last feeding the animals were anesthetized with nembutal and exsanguinated. Immediately after sacrifice, the livers were washed with a normal saline solution until

[1] Amidex. Corn Products Refining Company, New York, N. Y.
[2] Primex.

free from red cells and were dried on filter paper and weighed. The liver was then homogenized with about 70 ml of 0.85% NaCl solution in a micro Waring Blendor and made up to a volume of 250 ml. Aliquot samples (1.0 ml) were taken for determination of the total nitrogen by the micro Kjeldahl method.

The results of a typical experiment (see table 3) demonstrate that our samples of lactalbumin and casein were defi-

TABLE 3

*Liver protein regeneration properties* [1]

| CATEGORY OF INTEREST | DIET FED | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | Fasting | Egg white | Lactal-bumin | Whole egg | Casein | Wheat gluten |
| Mg liver N per 100 gm body weight [2] | 122±1.2 | 91±1.6 | 105±1.1 | 125±1.8 | 109±1.8 | 117±1.6 | 107±1.4 |
| Average body weight before fasting (gm) | 228 | 211 | 222 | 223 | 221 | 217 | 222 |

[1] The authors are indebted to Mrs. Lois Barrows for her technical assistance in the assay.

[2] S.E. = Standard error.

nitely superior to our whole egg or our egg white for the regeneration of liver proteins. This is not in complete accord with the findings of Harrison and Long ('45). Their data revealed no significant difference in the increase of liver nitrogen between rats fed with whole egg proteins and animals given casein or lactalbumin at a similar level. This discrepancy may be due to the difference in the samples used. It is also interesting to note that wheat gluten is not inferior to the egg proteins, according to this particular test.

*Repletion with different proteins*

In order to examine the plasma protein regeneration properties of the various proteins, the protein-depleted dogs were fed with diets containing one of the 5 different test proteins so that each animal received 0.35 gm of nitrogen and 80 cal. per kilogram body weight per day for a period of at least 4 weeks. The exception was that 0.6 gm of wheat gluten nitrogen was given instead of 0.35 gm, because of its low biological value. Determinations of total circulating plasma proteins, as well as albumin and globulins, were made before feeding and again two and 4 weeks after feeding (table 4). In summarizing our results we assumed the total circulating proteins, albumin or globulins immediately before feeding, to be 100%. Any change in plasma proteins due to protein feeding is expressed in terms of this figure.

*Total circulating plasma protein*

Our data (see table 4) demonstrate that supplementation of the protein-free diet with any one of the 5 proteins or two hydrolysates stimulated an increase in the total circulating plasma proteins, although the difference in the per cent increase over the depleted state was not great regardless of the nutritive values of the proteins fed. This fact in itself makes the quantitative determination of the increase in total circulating proteins not a very precise measure of the nutritive values of proteins. The lack of correlation is demonstrated by the fact that lactalbumin, with a nitrogen balance index of unity, did not promote the regeneration of plasma proteins as effectively as casein or the whole egg proteins having nitrogen balance indexes of 0.80 and 0.95, respectively. Egg white, which has the highest biological value among the 5 proteins, failed to bring about regeneration of plasma proteins to the same extent as casein even when feeding was continued for 8 weeks. A comparison between the whole proteins and their hydrolysates shows that the plasma protein regeneration property of casein was not improved by tryptic digestion,

TABLE 4

*Effects of oral feeding of various proteins on total albumin and globulins of dogs depleted in proteins* [1]

| PROTEIN FED | NO. DOGS USED | TOTAL CIRCULATING PROTEIN AFTER FEEDING FOR [2] | | TOTAL CIRCULATING ALBUMIN AFTER FEEDING FOR [2] | | TOTAL CIRCULATING GLOBULINS AFTER FEEDING FOR [2] | | A/G AFTER FEEDING FOR | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 wks. | 4 wks. | 2 wks. | 4 wks. | 2 wks. | 4 wks. | 0 wks. | 2 wks. | 4 wks. |
| Egg white | 10 | 117 ± 5 | 132 ± 17 | 117 ± 8 | 133 ± 12 | 118 ± 5 | 126 ± 16 | 0.28 | 0.27 | 0.26 |
| Lactalbumin | 5 | 115 ± 10 | 128 ± 10 | 185 ± 21 | 252 ± 23 | 100 ± 9 | 100 ± 9 | 0.22 | 0.42 | 0.58 |
| Whole egg | 5 | 148 ± 7 | 154 ± 7 | 206 ± 21 | 289 ± 22 | 138 ± 6 | 129 ± 7 | 0.19 | 0.28 | 0.43 |
| Casein | 4 | 138 ± 9 | 141 ± 5 | 177 ± 17 | 216 ± 19 | 130 ± 8 | 124 ± 4 | 0.25 | 0.33 | 0.39 |
| Wheat gluten | 5 | 135 ± 4 | 135 ± 4 | 162 ± 6 | 168 ± 7 | 130 ± 5 | 129 ± 5 | 0.17 | 0.21 | 0.22 |
| Lactalbumin hydrolysate | 6 | 141 ± 8 | 145 ± 6 | 230 ± 5 | 362 ± 8 | 115 ± 6 | 98 ± 7 | 0.21 | 0.44 | 0.85 |
| Casein hydrolysate | 5 | 138 ± 7 | 150 ± 3 | 223 ± 4 | 238 ± 8 | 119 ± 11 | 124 ± 10 | 0.22 | 0.41 | 0.51 |

[1] All the proteins were given at a level of 0.35 gm N/kg body weight/day except wheat gluten, which was given at a 0.6 gm level.

[2] The total circulating proteins before feeding are taken as 100%. The figures after the ± signs are standard errors.

while the tryptic digest of lactalbumin was definitely superior in this respect to the whole protein. However, it should also be pointed out that casein is rapidly hydrolyzed by the proteolytic enzymes normally present in the digestive tracts of the animals, whereas lactalbumin is not.

*Total circulating albumin*

Since animals suffer a severe loss in the total circulating albumin as a result of depletion, it seems reasonable to expect that on repletion the albumin will be regenerated in preference to other plasma proteins. The data demonstrate that feeding each of these 5 proteins brought about a marked increase in albumin, ranging from 191% for lactalbumin to 117% for egg white after two weeks of feeding. The latter protein is surprisingly ineffective for the purpose of plasma albumin regeneration. It is interesting to note that statistically the rates of regeneration of albumin by the hydrolysates were significantly greater than those of the corresponding proteins (191% against 230% for lactalbumin and its hydrolysate; 177% against 223% for casein and its hydrolysate). After 4 weeks of feeding the regeneration of albumin continues except with wheat gluten, egg white, and casein hydrolysate.

*Total circulating globulins*

An examination of our data on globulin regeneration shows that protein supplementation brought about an increase in the total circulating globulins except with lactalbumin or its hydrolysate. Since protein depletion results in only a very slight decrease in the globulin content, it is to be expected that on repletion the increase of globulins is likewise less marked than that of the albumin. Our data do not demonstrate the existence of any significant difference in the regeneration of globulins among the various proteins. The most pertinent information gained in this study, therefore, is the lack of ability of lactalbumin or its hydrolysates to regenerate globulins, although they surpass other proteins in stimulating the

production of albumin. The results of electrophoretic analyses of various globulin fractions in the plasma of protein-depleted dogs before and after feeding the test proteins and hydrolysates are shown in table 5. It should be emphasized that the electrophoretic analyses of the individual globulin fractions are more subject to error than the albumin (Chow et al., '45; Petermann et al., '47). The data, nevertheless, demonstrate that the total circulating alpha globulins were slightly increased by feeding egg white proteins and wheat gluten, but

TABLE 5

*Effects of oral feeding of different proteins on the various globulin fractions in the plasma of protein-depleted dogs*

| PROTEIN FED | NUMBER DOGS USED | TOTAL CIRCULATING GLOBULINS AFTER 4 WEEKS OF FEEDING [1] | | |
|---|---|---|---|---|
| | | alpha (1 and 2) | gamma | other globulins |
| Egg white | 6 | 125 ± 14 | 110 ± 14 | 133 ± 19 |
| Lactalbumin | 4 | 78 ± 11 | 73 ± 9 | 113 ± 10 |
| Whole egg | 5 | 103 ± 14 | 136 ± 5 | 139 ± 11 |
| Casein | 6 | 111 ± 7 | 125 ± 8 | 131 ± 5 |
| Wheat gluten | 5 | 131 ± 8 | 125 ± 6 | 130 ± 5 |
| Lactalbumin hydrolysate | 6 | 73 ± 16 | 130 ± 16 | 108 ± 17 |
| Casein hydrolysate | 5 | 92 ± 16 | 172 ± 5 | 137 ± 7 |

[1] The total circulating globulins before feeding are taken as 100%. The figures after the ± signs are standard errors.

were decreased by feeding lactalbumin hydrolysate. Other proteins did not cause any significant change in this particular fraction. The gamma globulin, in which the antibodies lie, is also significantly increased by protein supplementation. Other globulin fractions (i.e., all other globulins excluding the alpha and gamma forms) were increased following the addition of the proteins to the protein-free diet except in the cases of lactalbumin and its hydrolysate.

The albumin to globulin ratios, as determined electrophoretically on samples of plasma taken immediately before

feeding and 4 weeks after feeding, are given in table 4. Our data demonstrate that this initial ratio varied between 0.17 and 0.28, with an average of 0.23. Repletion with the two hydrolysates or with lactalbumin brought the ratio to approximately normal.[3] Casein and the whole egg proteins were less effective. The A/G values of the plasmas of the dogs receiving egg white and wheat gluten proteins were not increased.

## DISCUSSION

The fact that various food proteins differ qualitatively in their ability to promote synthesis of plasma proteins has been established by investigators at Rochester (Holman et al., '34; Madden et al., '37, '38; McNaught et al., '36; Pommerenke et al., '35) Yale (Melnick et al., '36a, '36b, '37) and Columbia (Weech and Goettsch, '38a, '38b, '39). Table 6 was prepared in order to present a comparison of the data reported by these investigators with those we have obtained. The proteins are arranged according to the descending order of the nitrogen balance indexes, with egg white (index of 1.1) at the top and wheat gluten (index 0.3) at the bottom. According to the plasmapheresis technique, casein was definitely inferior to both lactalbumin and egg white. Determination with the Weech and Goettsch partial depletion technique ('38a), and on the basis of the increase of chemically estimated albumin showed that lactalbumin and egg white proteins were superior to casein and wheat gluten. In terms of the increase of total circulating proteins of the more severely depleted dogs, all 5 proteins were more or less alike. In terms of electrophoretic albumin, whole egg proteins and lactalbumin were superior to casein, wheat gluten and egg white. However, in terms of globulin regeneration lactalbumin or its hydrolysate was the most ineffective. In examining the results given in table 6 it is well to note that the methods of assay

[3] In our experience with more than 50 analyses involving 45 dogs which had been on a commercial dog food (Friskies, Albers Milling Company, Peoria, Ill.) and whole egg with occasional liver supplement for 4 to 6 weeks, the A/G ratio was $0.64 \pm 0.026$.

were entirely different. As pointed out by Weech ('42), the plasmapheresis technique (Madden and Whipple, '40) measures *total capacity* for the formation of serum protein as opposed to the depletion technique (Weech) which measures relative differences in rate of the formation of albumin as estimated by the salt fractionation method. In the present study the animals were put on a protein-free diet for a period

TABLE 6

*A comparison of the plasma protein regeneration properties of different proteins measured with various techniques*

| SOURCE OF PROTEIN | SERUM PROTEINS PRODUCED BY FEEDING 100 GM DIETARY PROTEIN DURING PLASMAPHERESIS [1] | "CHEMICAL ALBUMIN" RISE IN THREE WEEKS (PARTIAL DEPLETION TECHNIQUE OF WEECH) [2] | DEPLETION BY PROTEIN-FREE DIET FOR 6 TO 8 WEEKS: INCREASE IN TOTAL CIRCULATING [3] | | |
|---|---|---|---|---|---|
| | | | Protein | Albumin | Globulin |
| | *gm* | *gm/100 ml* | % | % | % |
| Egg white | 17 | 0.616 | 130 | 160 | 130 |
| Lactalbumin | 18 | 0.773 | 130 | 260 | 100 |
| Whole egg | .. | 0.602 | 150 | 290 | 130 |
| Casein | 10 | 0.425 | 140 | 220 | 120 |
| Wheat gluten | .. | 0.207 | 140 | 170 | 130 |

[1] The figures refer to grams of serum proteins produced by feeding 100 gm of dietary protein.

[2] The figures refer to 0.15 plus the rise in albumin concentration (in gm/100 ml) in the serum during the week in which the food was fed. The constant, 0.15, is to be regarded as an allowance for maintenance.

[3] The figures refer to increase in total circulating proteins expressed as per cent of the initial values before feeding, i.e., 100% for depleted animals.

of 6 to 8 weeks instead of three weeks (Weech's procedure), so that the plasma proteins reached a level of 4.0 to 4.5 gm %.

In addition to differences in the nutritional states of the dogs used, it must be mentioned that the 5 test proteins under comparison are not chemical entities. The nutritive quality of samples of lactalbumin might differ, depending on the process used in preparing them. In our experience, some commercial samples of lactalbumin gave nitrogen balance indexes ranging from 0.8 to 1.0. In the case of egg white, the situa-

tion is further complicated by the presence of the trypsin inhibitor (Harte, '45) and avidin, which might make some species of test animals deficient in biotin.

In the present paper are presented results of the measurement by different methods of the nutritive qualities of identical samples of 5 proteins. Hence, any variation in our results cannot be attributed to differences in the samples of proteins used. The results demonstrate that, in general, the commonly-used methods of measurement show a marked similarity in results except as regards liver protein regeneration and plasma protein regeneration properties. In the latter case, the criterion must specify whether the increase is of albumin or globulins.

The marked difference in the plasma protein regeneration properties of lactalbumin and casein is confirmed in whole proteins as in the hydrolysates, although the experiments were not designed to elucidate the explanation for this difference.

It is also of interest to note that there appears to be a significant difference between lactalbumin and its hydrolysate with respect to rate of albumin regeneration. It is conceivable that the hydrolysate is more efficiently utilized because of the availability of all the essential amino acids at one time. Digestion of lactalbumin by the animals not only takes time but may release different essential peptides at different rates (Melnick et al., '46). On the other hand, a protein like casein is readily hydrolyzed in the intestines and therefore it is reasonable to believe that it is absorbed as readily as its hydrolysate.

## SUMMARY

The nutritive properties of 5 proteins (whole egg, egg white, lactalbumin, casein and wheat gluten) as well as two hydrolysates of casein and lactalbumin present in commonly consumed diets were measured with respect to their ability to support nitrogen balance, to promote growth, to replace body tissues, to regenerate liver proteins, and to stimulate the production of plasma proteins. It was found that, in general,

a parallelism exists among the results arrived at by the test methods used except for egg white, which is superior to the 4 other proteins according to the first three tests but not according to the last two.

Our data again demonstrate that lactalbumin or its hydrolysate favors the regeneration of albumin in protein, whereas other proteins will regenerate globulins as well. Examination of the A/G ratio after repletion demonstrates that the ratio returned to normal only after the feeding of lactalbumin, its hydrolysate, and casein hydrolysate.

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# PTEROYLGLUTAMIC ACID BALANCE STUDIES ON MONKEYS[1]

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TWO FIGURES

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The urinary excretion of pteroylglutamic acid (PGA) by normal human subjects receiving a mixed diet with no PGA supplement is quite low, being usually less than 5 μg per 24 hours (Johnson, Hamilton and Mitchell, '45; Steinkamp, Shukers, Totter and Day, '46; Swendseid, Bird, Brown and Bethell, '47; Jukes, Franklin, Stokstad and Boehne, '47). Following the oral administration of 5 mg of PGA to such normal subjects, 30 to 50% of the test dose appears in the urine within 24 hours (Steinkamp et al., '46; Swendseid et al., '47; Jukes et al., '47). Most of the excreted material appears in the urine within the first 4 hours following dosage (Steinkamp et al., '46). The urinary excretion of a group of 7 hospital patients with various blood dyscrasias, before PGA treatment, did not differ significantly from that of normal subjects; however, following a test dose of PGA, the urinary returns were less than 11% of the dose (Steinkamp et al., '46).

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At least half of the administered oral dose of PGA is therefore unaccounted for. We might speculate that it could: (1) remain unabsorbed and appear in the feces; (2) be destroyed in the intestinal tract; (3) be absorbed and stored in the tissues; (4) be absorbed and destroyed in metabolism; (5) be absorbed and converted into some metabolite which does not stimulate the growth of *Streptococcus faecalis* or *Lactobacillus casei* and in that form be excreted in the urine; or (6) be disposed of by a combination of the foregoing. It seemed feasible to test the first of these possibilities by balance experiments upon a suitable primate. Such studies have been made upon the rhesus monkey and are reported in this paper.

## EXPERIMENTAL

Young rhesus monkeys (2.1–2.9 kg) were housed in steel metabolism cages equipped with funnel-shaped stainless steel pans for the collection of urine. Routine hematological studies were made at weekly intervals. After a preliminary period of observation the animals were given the PGA-deficient diet described by Day and Totter ('47; see p. 317). This diet, if unsupplemented with PGA or some crude source of the vitamin, will eventually result in anemia, leukopenia, ulceration of the gums, diarrhea, and death. The anemia and leukopenia so induced respond dramatically to either pteroyltriglutamic acid (Day, Mims and Totter, '45) or to synthetic PGA (Totter, '46). Although the amount of this ration fed per monkey per day contains approximately 20 μg of PGA, the diet consistently produces the deficiency syndrome. It may, in fact, be more suitable than highly refined diets for the production of PGA deficiency, since diets containing purified foodstuffs only have been shown to be deficient in another factor required by the monkey (Cooperman et al., '46).

In the experiments here described, daily urine and fecal collections were made for microbiological assay. The urine was collected under benzene, diluted to a convenient volume, and stored in a refrigerator. The feces were collected,

weighed and mixed, and a weighed sample of them was homogenized in a Waring Blendor and stored in the refrigerator. The microbiological assays (Mitchell and Snell, '41) employed *Streptococcus faecalis* (American Type Culture Collection no. 8043), using synthetic PGA as a standard. Turbidimetric readings were made with a Coleman spectrophotometer. In one series of assays, to be referred to later, simultaneous determinations were also made using *Lactobacillus casei*. The urines were assayed for free PGA only. Assays were made on the feces for both free and conjugated PGA; for the conjugated PGA, conjugase preparations from rat liver (Mims, Totter and Day, '44), chicken pancreas (Laskowski et al., '45) and hog kidney (Bird et al., '45) were employed. The highest accurate value obtained with the three enzymes was used. The period of collection for each experiment was three weeks or longer.

In the balance experiments here reported, the following materials were fed to monkeys, as daily supplements to the deficient diet, under suitably controlled conditions: 100 μg of synthetic PGA; 1000 μg of synthetic PGA; 100 μg of pteroic acid; 2.5 gm of yeast extract,[2] which contained approximately 100 μg of PGA, largely as the conjugate. Balance studies were also made on a control monkey receiving no supplement to the deficient diet. The 100 μg level was chosen as a daily supplement because this appears to be approximately the minimum protective dose of PGA for a young monkey weighing 2–3 kg. Although, on the basis of microbiological assays of materials previously shown to protect the monkey, Day and Totter ('47) tentatively set the minimum daily protective level at 40 to 80 μg PGA as supplement to the deficient diet, subsequent unpublished experiments with synthetic PGA have indicated that 100 μg is probably the minimum daily dose necessary to protect against deficiency manifestations. The monkey which was given 2.5 gm of yeast extract likewise received the minimum protective amount of PGA, in the form of the conjugate equivalent to about 100 μg of PGA.

[2] Difco.

Although under certain circumstances the monkey may not satisfactorily utilize the conjugate, an amount of conjugate equivalent to 100 μg of PGA was found completely protective when fed in the form of yeast in long-term experiments (Day and Totter, '47, pp. 319–320).

The data obtained were treated statistically by the analysis of variance method (Snedecor, '46).

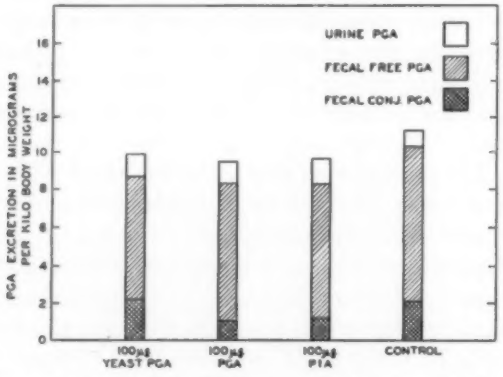


Fig. 1 Average daily excretion data, expressed in terms of microbiologically equivalent amounts of PGA, on monkeys receiving daily the amounts of the materials indicated below the respective bars.

## RESULTS AND DISCUSSION

The monkeys receiving 100 μg of PGA, 1000 μg of PGA, and 2.5 gm of yeast extract maintained normal blood pictures throughout the studies, as would be expected. During the short period of the balance study, the monkey receiving 100 μg of pteroic acid was also free of deficiency manifestations; although we assume that pteroic acid — at this level at least — would not protect the monkey, the experiment was not continued long enough to prove this point.

Figure 1 presents in graphic form the average daily excretions of PGA (i.e., substances stimulating the growth of *Streptococcus faecalis*) by monkeys fed 2.5 gm of yeast [3] (con-

[3] See footnote 2, p. 805.

taining conjugate equivalent to 100 μg of PGA), 100 μg of synthetic PGA, and 100 μg of synthetic pteroic acid (PtA); and by a control monkey receiving no supplement to the deficient diet. In this figure the excretion data are plotted per kilogram of body weight. The control monkey was somewhat larger (2.96 kg) than the other animals (2.08–2.43 kg). When expressed in terms of total outputs, the deficient control excreted significantly greater amounts than the other monkeys. It therefore seemed more valid to make comparisons per unit of body weight. Expressed in this way, as is shown in the figure, there appear to be no significant differences among the 4 animals as regards urinary excretion, fecal excretion, and total excretion. A statistical study of the data has confirmed the impression that the differences are not significant.

Several conclusions seem immediately apparent and fully justified on the basis of this experiment. The minimum daily protective dose of synthetic PGA (100 μg), when given by mouth, does not significantly increase either the urinary or the fecal excretion of microbiologically active substances. It is thus obvious that at this level of intake the PGA does not pass through the intestinal tract unchanged; from these data we cannot conclude whether it is (1) altered or destroyed in the intestinal tract or (2) absorbed from the tract.

Pteroic acid, when fed at the 100 μg per day level, did not appreciably increase the excretion of active substances in either the urine or the feces. Here again, we cannot decide on the basis of these data whether the pteroic acid was chemically altered in the tract and excreted in the feces in a form microbiologically inactive, or whether it was absorbed from the tract. In any event, it did not appear in the urine in measurable quantities, for the urinary level of microbiologically active material was not increased. Furthermore, urine samples from this monkey and from the monkey receiving 100 μg of PGA were assayed with both *Lactobacillus casei* and *Streptococcus faecalis*. The assay values obtained with the two organisms were in substantial agreement for

both animals. This constitutes additional evidence that orally administered pteroic acid, at this level of intake, does not appear in the urine in measurable quantities.

The conclusions to be drawn from the yeast experiment may be no less significant. The conjugate supplied by 2.5 gm of yeast extract did not alter the total fecal output or the conjugate fecal output to any important extent. Actually the data (fig. 1) show a fecal conjugate excretion by the yeast monkey twice that of the PGA monkey. It must be remembered, however, that the measurement of the conjugate, even in the hands of a skilled technician, is subject to several cumulative errors with the methods now available. Because of these possible errors and the small quantity of conjugate in the fecal specimens, we attach no great significance to these apparent differences in conjugate content. Whether such differences are significant or not, this point stands out clearly: the average daily conjugate content of the feces of the monkey receiving yeast extract was only 5% of the yeast PGA conjugate fed. Quite evidently, crude yeast extract conjugate does not pass through the intestinal tract of the monkey unchanged.

A general conclusion seems warranted from this experiment. The young rhesus monkey on a PGA-deficient diet continues to excrete a limited amount of material having microbiological activity like that of PGA. In this experiment the daily output in the urine averaged about 1 μg, and in the feces about 10 μg, per kilogram of body weight. These excretion levels must be looked upon as basal levels. They were not increased by an oral intake of synthetic PGA sufficient to supply the minimum requirement of the monkey (100 μg per monkey per day), nor by 100 μg of pteroic acid, nor by yeast conjugate equivalent to 100 μg of PGA.

This concept of a continuous basal urinary and fecal excretion of PGA by the monkey must not be interpreted as implying a rigidly *constant* excretion. Such is not the case. Repeated observations of a given animal receiving the same diet for a long period have shown significant alterations in

output from time to time. Whether these changes are the result of seasonal or temperature changes, minor differences in diet constituents from batch to batch, or to other factors, we cannot say at this time. These findings do emphasize the importance of rigid control in such balance experiments, either by the use of simultaneous experiments or by consecutive experiments on a single animal.

Figure 2 represents the data from an experiment in which the intakes were 1000 and 100 μg of synthetic PGA respec-

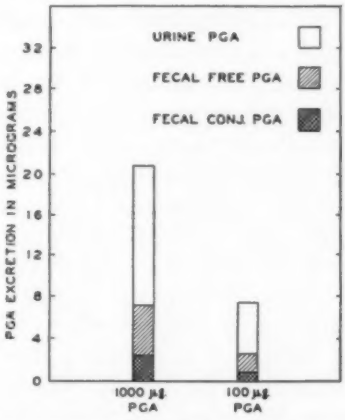


Fig. 2 Average daily excretion data, expressed in terms of microbiologically equivalent amounts of PGA, on a monkey receiving 1000 μg and 100 μg of synthetic PGA daily, in successive experimental periods.

tively, the former value a 10-fold increase over the minimum daily requirement of the monkey. These were successive studies on the same animal, and since the monkey did not change weight during the series, comparisons are valid without taking body weight into consideration. It is immediately apparent from an inspection of the figure that the outputs in both urine and feces were significantly greater during the 1000 μg period than during the 100 μg period. Statistical treatment of the data confirms the significance of these differences. Viewing our findings superficially, we would con-

clude that a 10-fold increase in intake resulted in a three-fold increase in output. Examining the data in the light of the experiment summarized in figure 1 and similar unpublished experiments, however, we may properly conclude that the output of the animal during the 100 μg intake period was the "basal" output, which would have been essentially the same had no PGA been added to the deficient diet. If we subtract this "basal" urinary output (4.8 μg) from the average urinary output during the 1000 μg period, we find that the increase in urinary output was approximately 10 μg, which is only about 1% of the daily dose. One might speculate that this low urinary return was the result of low tissue levels of PGA, in which case the feeding of large daily doses over a long period should result in tissue saturation and an increase in urinary output. However, no such increase has been observed in a monkey which has received 1 mg of PGA daily for 80 days. This low percentage of urinary excretion is in sharp contrast to the response of the normal adult human, who excretes in the urine 30–50% of a 5 mg oral test dose of PGA within 24 hours. This difference may be related to the peculiar susceptibility of the young rhesus monkey to PGA deficiency.

It is also clear from this experiment that orally administered synthetic PGA does not, to any important extent, pass unchanged through the intestinal tract of the monkey; although the higher level of feeding resulted in an increased fecal output, that daily fecal increase was less than 1% of the daily dose.

The fate of the other 98% of the 1000 μg dose of PGA is yet to be elucidated. We cannot conclude from these experiments whether it is altered or destroyed in the intestinal tract, whether it is absorbed and stored in the tissues, or whether it is absorbed and then altered or destroyed in metabolism.

## SUMMARY

Young rhesus monkeys were given a diet known to produce the anemia, leukopenia, and other manifestations of pteroyl-

glutamic acid (PGA) deficiency. Daily urine and fecal collections were made and these excreta assayed for PGA, using *Streptococcus faecalis*. Suitable conjugase preparations were employed in the fecal analyses. Each experimental period lasted 21 days or longer.

The following daily additions were made to the diets of such animals, under suitably controlled conditions: 100 μg of synthetic PGA (which is the approximate minimum daily requirement); 100 μg of synthetic pteroic acid; 2.5 gm of yeast extract, containing approximately 100 μg of PGA, largely in the form of the conjugate. The average daily urinary and fecal PGA excretions of these monkeys, when expressed on a body weight basis, were not significantly different from the excretions of a control monkey receiving the deficient diet only. In this series the average daily urinary outputs were approximately 1 μg, and the average fecal outputs were between 8 and 10 μg, expressed in terms of synthetic PGA per kilogram of body weight. These may be considered "basal" levels of excretion.

It is concluded that neither synthetic PGA, synthetic pteroic acid, nor crude yeast PGA conjugate, when fed at the 100 μg level, pass through the intestinal tract of the young rhesus monkey unchanged.

Increasing the daily intake of synthetic PGA to 1000 μg resulted in a three-fold increase in urinary and fecal output over the "basal" level. Notwithstanding, the actual urinary increase was only about 1% of the oral dose and the fecal increase much less than 1% of the dose. Obviously, even at high levels of intake, synthetic PGA does not, to any significant extent, pass through the intestinal tract of the monkey unchanged.

The low percentage urinary return of large daily oral doses of synthetic PGA by the monkey is in sharp contrast to the response of the normal adult human, who excretes 30–50% of a 5 mg oral dose within 24 hours.

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# THE ROLE OF THE TIME FACTOR IN FEEDING SUPPLEMENTARY PROTEINS

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It has been demonstrated in previous experiments (Geiger, '47; Cannon et al., '47; Schaeffer and Geiger, '47), that incomplete amino acid mixtures cannot be stored in the animal organism, and that protein synthesis occurs only when all the essential building stones are available simultaneously. These results were recently confirmed by Harte, Travers and Sarich ('48), who demonstrated that alternate feeding of wheat gluten and a protein hydrolysate resulted in significantly poorer growth than that obtained when both sources of nitrogen were fed together. These results are further supported by the experiments of Yeshoda and Damodaran ('47), who found that injections of tryptophane into animals on tryptophane-free diets had a hemopoietic effect but failed to prevent the loss of body weight. Finally, Pearce, Sauberlich and Baumann ('47) have shown for mice, and Sauberlich and Baumann ('48) for rats, that urinary amino acid losses are increased when the animals are fed incomplete amino acid mixtures. In all these experiments, only amino acids themselves or amino acid mixtures were investigated. The results, therefore, are primarily of theoretical interest and their practical value is restricted to those instances when mixtures of the amino acids are fed or injected. The proteins actually present

in food differ from the amino acids in that they are slowly digested and absorbed and therefore it seemed advisable to ascertain whether the findings discussed above could be extended to the dietary proteins; hence the present investigation of the metabolic fate of the proteins which lack one or more essential building stone and therefore do not promote growth satisfactorily.

In order to study this problem it was decided to select pairs of proteins which, fed separately, do not promote growth, but which, when given together, supplement each other's amino acid deficiencies. By alternately feeding such protein pairs the author intended to duplicate the conditions which obtained in earlier experiments with delayed supplementation of amino acids.

## METHODS

The composition of the basal diet was as follows: Corn starch, 3050 gm (78.3%); rice bran concentrate, 400 gm (10.26%); cottonseed oil, 200 gm (5.13%); U.S.P. salt mixture, 200 gm (5.13%); fish oil (1 gm contains 2,000 I.U. vitamin A and 400 I.U. vitamin D) 50 gm (1.27%); riboflavin, 75 mg; Ca pantothenate, 150 mg; choline chloride, 2.5 gm. The protein sources used and their respective nitrogen contents in per cent were as follows: cultured yeast (Strain G),[1] N (Kjeldahl) 6.9; wheat gluten,[2] N (Kjeldahl) 12.14; blood protein,[3] N(Kjeldahl) 13.43.

Male Sprague-Dawley rats weighing from 45 to 50 gm were used. The animals were placed in individual cages. Body weight and food consumption were determined daily. A detailed description of the technic of feeding has been given in an earlier paper (Geiger, '47).

The rats of the different groups received food from 8:00 A.M. to 6:00 P.M. and from 8:00 P.M. to 6:00 A.M. The feeding cups were removed for two-hour intervals between the feeding periods.

[1] Anheuser Busch.
[2] Pure Gluten Food Co.
[3] Viobin Corp.

## EXPERIMENTAL

The results of these experiments are presented in table 1.

TABLE 1

*Growth of rats during 16 days on feeding of supplementary pairs of proteins*

| PLAN OF FEEDING | PAIRS OF SUPPLEMENTARY PROTEINS | | |
|---|---|---|---|
| | Wheat gluten + blood protein | Yeast + blood protein | Wheat gluten + yeast |
| | *gm* | *gm* | *gm* |
| | Group III | Group III | Group III |
| Fed together | 19 | 16 | 24 |
| | 22 | 15 | 22 |
| | 18 | 17 | 28 |
| | 30 | 22 | 30 |
| | Group IV | Group IV | Group IV |
| Fed separately | 3 | —9 | —4 |
| | 2 | —3 | 3 |
| | —2 | —5 | 4 |
| | —4 | 2 | 6 |

### *Experiments with wheat gluten and blood protein*

The first pair of supplementary proteins studied consisted of wheat gluten and blood protein. Wheat gluten is deficient in lysine (Mitchell and Block, '46), while blood protein is especially low in isoleucine (Albanese, '45).

Four groups of male rats were used, each consisting of 4 animals of practically equal weight (45–50 gm). Group I received a diet containing 9% blood protein, group II one containing 9% wheat gluten, and group III a ration containing 4.5% blood protein and 4.5% wheat gluten; group IV was fed a diet containing 9% blood protein for 10 hours and, for the next 10-hour feeding period, one containing 9% wheat gluten.

The animals of groups I and II showed no growth for a period of 16 days, indicating that neither blood protein nor wheat gluten by itself promotes growth when fed at a 9% level. During the 16-day period the animals of group III

gained 19, 18, 22, and 30 gm, indicating that blood protein and wheat gluten fed together promote growth, i.e., that they supplement each other's amino acid deficiencies. Groups III and IV were subjected to paired feeding; their total food and protein intakes were practically the same, being 130 ± 5 gm total food intake per animal for 16 days. In spite of this, the animals of group IV which were fed rotating diets containing blood protein and wheat gluten showed no appreciable growth. One animal gained 3 gm; the second, 2 gm; the third and 4th animals lost 2 and 4 gm, respectively.

These experiments indicate that the two proteins under examination exert their complementary influences only when fed simultaneously.

### *Experiments with yeast and blood protein*

In the second group of experiments yeast was used as a protein complementary to the blood protein. The method of feeding was the same as that used in the preceding experiments with wheat gluten.

The animals of group I received 9% blood protein and of group II 9% yeast protein in their diets. Neither of these groups showed increase in growth. The animals of group III, receiving a diet containing 4.5% blood and 4.5% yeast protein, gained 16, 15, 17 and 22 gm. In group IV, where diets containing 9% yeast and 9% blood protein were fed in rotation at 10-hour intervals for 16 days, no growth occurred. The changes in weight were −9, −3, +2 and −5 gm for the 4 groups, respectively. The paired-fed rats of groups III and IV consumed 145 ± 12 gm of food each for the 16-day period.

These experiments indicate that yeast and blood proteins supplement each other's growth-promoting properties only when fed simultaneously.

### *Experiments with yeast and wheat gluten*

In a third group of experiments we selected, as complementary proteins, yeast and wheat gluten. According to the

data of Block and Bolling ('45), yeast contains 6% lysine while wheat gluten contains only 1.1%. Methionine, on the other hand, is present in high concentration in wheat gluten (5.5%), and is low in yeast (2%). In control experiments it was shown in confirmation of the data of Klose and Fevold ('45), that yeast protein fed on a 9% basis does not promote growth in infant rats; wheat gluten was likewise unable to promote growth when fed on this basis.

In order to investigate their complementary effects on each other, a diet was fed containing 4.5% wheat gluten and 4.5% yeast protein to 4 rats in group III. These animals showed a very good growth, gaining 24, 22, 28 and 30 gm, respectively, during the 16-day experimental period. To group IV was fed alternately, for two 10-hour periods, a diet containing 9% wheat gluten, and one containing 9% yeast protein. The change in body weight was insignificant; one animal lost 4 gm and the others gained 3, 4 and 6 gm, respectively. The total food consumption for each animal subjected to paired feeding was 222 $\pm$ 8 gm.

These experiments indicate that when yeast protein and wheat gluten protein are fed together they supplement each other's amino acid deficiencies but that feeding these proteins at separate times interferes with their effective complementary actions.

It may therefore be concluded that, as in our earlier experiments with amino acid mixtures, the building stones of deficient proteins cannot be stored, and that the delayed provision of the missing factors is ineffective not only when they are fed as free amino acids but also when they are supplied in the form of proteins.

A consequence of these experiments seems to be the conclusion that supplementary proteins of individually low biological value must be fed together in order to achieve their optimum utilization.[4]

After presenting these results at a meeting of the American Association for the Advancement of Science, San Francisco,

[4] Editorial, Nutrition Reviews, *5:* 316 (1947).

1948, our attention was called to a paper by Henry and Kon ('46), who found that milk and potato, and bread and cheese, exhibit supplementary relationships only if fed together and not if fed separately on alternate days. These results are in complete agreement with our conclusions. It is, however, possible that in the experiments of Henry and Kon, where only highly complex foods such as bread, cheese or potatoes were investigated, some factors other than protein may be at least partially responsible for the results.

## SUMMARY

The question was investigated of whether supplementary proteins of low biological value promote the growth of infantile rats when fed individually at different times.

It was found that yeast, blood and wheat gluten proteins do not promote growth when fed individually at a 9% level. Diets containing wheat gluten + blood protein, or yeast + blood protein, or yeast + wheat gluten protein, have satisfactory growth-promoting properties. If, however, these same pairs of proteins are fed separately with lapses of time between feedings, they do not supplement each other, as is indicated by the lack of resulting growth.

These experiments show that delayed provision of the missing essential amino acids is ineffective, not only when fed as the amino acids themselves (Geiger, '47), but also when they are supplied in the form of proteins.

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## BORDEN AWARD IN NUTRITION

Nominations are solicited for the 1949 Award of $1000, and a gold medal made available by the Borden Company Foundation, Inc. The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute in the spring of 1949. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1949. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

JAMES M. ORTEN
*College of Medicine,*
*Wayne University,*
*Detroit, Michigan*

CHAIRMAN, NOMINATING COMMITTEE

## MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1949 Award of $1000, established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute in the spring of 1949.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year, January 1 to December 31, the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1948 must be in the hands of the Chairman of the Nominating Committee by January 15, 1949. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

HAROLD H. WILLIAMS
*Cornell University, Ithaca, N. Y.*

CHAIRMAN, NOMINATING COMMITTEE

## OSBORNE AND MENDEL AWARD

Nominations are invited for the Osborne and Mendel Award of $1000, established by the Nutrition Foundation, Inc., for the recognition of outstanding accomplishments in the general field of exploratory research in the science of nutrition. It shall be given to the investigator who, in the opinion of a Jury of Award, has made the most significant published contribution in the year preceding the annual meeting of the Institute, or who has published a series of contemporary papers of outstanding significance.

The Award will be presented at the annual meeting of the American Institute of Nutrition.

The recipient will be chosen by a Jury of Award of the American Institute of Nutrition. As a general policy, the Award will be made to one person. If, in the judgment of the Jury of Award, an injustice would otherwise be done, it may be divided among two or more persons. Normally preference will be given to research workers in the United States and Canada, but investigators in other countries, especially those sojourning in the United States or Canada for a period of time, are not excluded from consideration. Membership in the Institute of Nutrition is not a requirement for eligibility and there is no limitation as to age.

Nominations may be made by anyone. Nominations for the 1949 Award, accompanied by data relative to the accomplishments of the nominee, must be sent to the Chairman of the Nominating Committee before January 15, 1949.

D. W. Woolley
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Chairman, Nominating Committee

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